AN ABSTRACT OF THE THESIS OF

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Steven H. Strauss

We tested a synthetic bacterio-opsin (*bO*) gene derived from *Halobacterium halobium* for its effect on disease resistance in transgenic poplar hybrids, and assessed a genetic system for controlling expression of this gene. In tobacco the *bO* gene caused necrotic lesions similar to an induced hypersensitive response (HR), increased levels of several defense-related mRNAs, and enhanced resistance to pathogens (Mittler et al. 1995). Our goal was to test whether the *bO* gene could also promote the HR response and induce resistance against pathogens of poplar.

Using *Agrobacterium* transformation, we produced 35 transgenic lines within three hybrid poplar clones. Expression of the *bO* gene was under the control of the cauliflower mosaic virus (CaMV) 35S promoter and Ω translational enhancer, and the protein included a transit peptide to target it to the chloroplast. Transgenic lines were confirmed through their ability to root on kanamycin-containing medium, PCR amplification using *bO*-specific primers, and RNA blots probed with the *bO* gene.

Necrotic lesions induced by the *bO* gene were seen predominantly on the lower and older leaves of greenhouse- and field-grown trees, similar to that reported in tobacco. Most lines displayed low to moderate numbers of lesions (mean lesion index=6.6, coefficient of variation=106%); however, one line had a

very high lesion index (6-fold greater than the mean). Based on northern blots, *bO* expression among lines also varied widely (coefficient of variation=90%). We studied the effect of the *bO* gene on the expression of two types of defense-related genes, phenylalanine ammonia-lyase (*PAL1*), and two wound-inducible (*win*) chitinases, *win6* and *win8*. Because of the low constitutive expression of the chitinases in poplar, their expression was induced by mechanical wounding prior to RNA extraction. No significant change in expression was found for any of these genes associated with the expression of the *bO* gene, including the line with very high *bO* expression.

Resistance to different fungal pathogens was examined in the field and greenhouse after artificial inoculation. When field-grown trees were inoculated with fungal spores of *Venturia* leaf and shoot blight, *bO*-containing transgenics did not differ significantly from non-transgenic plants of the same clone (195-529). When clone 189-434, which is susceptible to the rust pathogen *Melampsora occidentalis*, was inoculated in the greenhouse, transgenic and control plants were also not significantly different in disease development. When all the lines were tested for susceptibility to two *Septoria* leaf and stem pathogens using *in vitro* inoculation, transgenics and controls were again similar in susceptibility. It was concluded that even though the *bO* gene was over-expressed and induced an HR-mimic, lesion phenotype in some lines, it did not induce broad-spectrum defense-related genes, and it failed to improve fungal disease resistance.

To test a system that might be suitable for regulating expression of disease resistance transgenes in poplar, we studied the copper-based gene activation system reported to be effective in tobacco. Using both histochemical and fluorometric assays with a *GUS* reporter gene, we found that transgenic poplar leaves with both control (promoter-*GUS* only) and induction (promoter-*GUS* plus the copper binding *ACE1* transcription factor) constructs displayed constitutive expression without the addition of exogenous copper (basal medium concentration was 0.032 μM $CuSO_4$). With the full induction construct, expression varied in a complex manner as a function of copper concentration (0 - 100 μM). It was concluded that

because of the presence of endogenous transcription factors or inducing ions that compete with copper, the system is unlikely to be useful for regulating gene expression in poplar leaves.

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Genetic Engineering of Disease Resistance in Poplar: Effects of Bacterio-opsin Over-expression and Analysis of a Copper-based System for Resistance-Gene Activation

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APPROVED

Signature redacted for privacy.

Major Professor, representing Forest Science

Signature redacted for privacy.

Chair of Department of Forest Science

Dean of Graduate School

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Rozi Mohamed, Author

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TABLE OF CONTENTS

Page

CHAPTER 1 1
INTRODUCTION AND LITERATURE REVIEW 1

IMPACT OF DISEASES ON POPLAR PLANTATIONS 1

IMPORTANT DISEASES OF POPLAR 3

Melampsora leaf rust 3
Venturia leaf and shoot blight 4
Septoria leaf spot and stem canker 5

DISEASE RESISTANCE 6

Natural mechanisms of disease resistance 7
Genetic engineering of disease resistance 8
Genetic engineering of HR-based resistance 11

GENETIC ENGINEERING OF POPLAR 13

Molecular genetics 13
Agrobacterium-mediated transformation 14

REGULATING TRANSGENE EXPRESSION 14

Inducible promoter systems 15
Copper-inducible gene expression system 16

CHAPTER 2 18
EFFECT OF OVER-EXPRESSION OF A BACTERIO-OPSIN GENE ON LESION FORMATION, GROWTH AND DISEASE RESISTANCE IN HYBRID POPLAR 18

ABSTRACT 18

INTRODUCTION 18

TABLE OF CONTENTS (Continued)

Page

MATERIALS AND METHODS 20

Bacterial plasmids and strains 20
Plant materials and transformation 21
Field trial and spontaneous lesion assessment 21
Fungal inoculation and disease assessment 23
Northern analysis and hybridization probes 25

RESULTS 27

Lesion formation and growth 27
Northern analysis 32
Effect of *bO* expression on resistance to fungal pathogens 35

DISCUSSION 38

Development of spontaneous lesions and effect on growth 38
Induction of defense-related genes 39
Resistance to pathogens 41

CHAPTER 3 44
EVALUATION OF A COPPER-INDUCIBLE GENE EXPRESSION SYSTEM IN TRANSGENIC POPLAR 44

ABSTRACT 44

INTRODUCTION 44

MATERIALS AND METHODS 46

Plasmid constructs 46
Poplar transformation and PCR analysis 46
Copper induction treatments 47
Measurement of GUS activity 48

RESULTS 49

DISCUSSION 51

TABLE OF CONTENTS (Continued)

Page

CHAPTER 4 54
CONCLUSIONS 54

MAJOR RESULTS 54

MAJOR CONCLUSIONS 55

FUTURE RESEARCH SUGGESTIONS 55

BIBLIOGRAPHY 58

APPENDICES 68

LIST OF FIGURES

Figure Page

1.1 The idealized mechanism of action of a copper-inducible gene expression system 17

2.1 Expression of spontaneous lesions on leaves from trees grown in the greenhouse and field 28

2.2 Mean lesion density for all transgenic and control lines as scored prior to inoculation study 29

2.3 Lesion density determined on leaf numbers 6 and 7 30

2.4 Lesion density determined from leaf numbers 2 to 7 31

2.5 Northern blot analysis of poplar transgenic lines expressing bacterio-opsin (*bO*) and phenylalanine ammonia-lyase (*PAL1*) genes. 33

2.6 Expression of several defense-related genes after induction via mechanical wounding 34

2.7 Lesion development prior to inoculation, and disease development following artificial inoculation with fungal spores causing *Venturia* shoot blight 36

2.8 Resistance of transgenic and non-transgenic poplar to *Septoria* leaf spot and stem canker diseases after *in vitro* inoculation. 38

3.1 Effect of copper ion concentration on *GUS* gene expression in two transgenic poplar lines (*P. trichocarpa* x *P. deltoides* clone 189-434) containing the full copper induction gene-construct 50

LIST OF APPENDICES

Appendix Page

A: PCR CONFIRMATION OF TRANSGENICS 69

B: TRANSGENIC LINE IDENTIFICATION NUMBERS 71

C: SPONTANEOUS LESION AND DISEASE ASESSMENT - LESION DEVELOPMENT AND RESISTANCE OF TRANSGENIC POPLARS TO *VENTURIA* LEAF AND SHOOT BLIGHT 72

D: ANALYSIS OF GROWTH RATE OF TRANSGENIC TREES IN FIELD TRIALS 81

E: QUANTIFICATION OF TRANSGENE AND *PAL1* EXPRESSION IN TRANSGENIC PLANTS 89

F: EXPRESSION AND QUANTIFICATION OF DEFENSE-RELATED GENES IN TRANSGENIC PLANTS 93

G: DISEASE ASSESSMENT - RESISTANCE OF TRANSGENIC POPLARS TO *MELAMPSORA* LEAF RUST 97

H: RESISTANCE OF *BO* TRANSGENIC POPLARS TO *SEPTORIA* LEAF SPOT AND STEM CANKER (U.S. FOREST SERVICE) 101

I: SUMMARY : EFFECT OF WATER STRESS ON LESION FORMATION IN TRANSGENIC POPLARS (U.S. FOREST SERVICE) 104

J: HISTOCHEMICAL AND FLUOROMETRIC GUS ASSAYS 109

K: CATALOGUE OF DNA, RNA, BLOTS, PRIMERS, AND COMPUTER RESOURCES REMAINING IN THE LABORATORY 115

LIST OF APPENDIX FIGURES

Figure Page

C.1 Lesion density and disease severity at Peavy Arboretum. 72

C.2 Lesion density and disease severity at Marchel Tract. 73

D.1 Net growth at Peavy Arboretum. 81

D.2 Net growth at Marchel Tract. 82

D.3 Net growth over both sites. 82

E.1 Relationship between *PAL1* and *bO* expression in transgenic and control lines as quantified from RNA blot A (left) and blot B (right) 89

E.2 Relationship between total lesion density and *bO* expression 92

F.1 Northern blot analysis of transgenic lines expressing *bO* and defense-related genes after induction via mechanical wounding. 93

F.2 RNA expression of several defense-related genes after induction via mechanical wounding. 94

G.1 *Melampsora* rust severity on transgenic lines and control plants 97

G.2 Clonal means for rust severity. Data are the mean rust severity from all transgenic lines and control in each clone 98

I.1 Lesion development in trees grown under WS or WW conditions over a 16-day period. 105

J.1 Histochemical *GUS* expression in transgenic poplar with the control construct containing *GUS* but lacking the *ACE1* regulatory element (A), and the full copper induction gene-construct containing *ACE1*(B) 110

LIST OF APPENDIX TABLES

Table Page

B.1 Transgenic poplar expressing the *bO* gene 71

C.1 Mean and SE of total lesion density (relative units) 73

C.2 Mean and SE of disease severity (relative units) 74

C.3 ANOVA table for *Venturia* blight severity 78

D.1 Mean and SE of net growth in log units [(lnVI2) – (lnVI1)] 83

D.2 Mean and SE of net growth in 100 cm^3 (VI2-VI1) 83

D.3 Mean and SE of net growth (ln) for 3 ramets from each line and the controls at Peavy Arboretum and Marchel Tract 84

D.4 ANOVA table for net growth 85

D.5 ANOVA table for net growth (after removing data from line 6) 85

E.1 Correlation analysis for variable *PAL1* and *bO* 90

F.1 RNA expression of several defense-related genes after induction via mechanical wounding as quantified from the above blot 94

F.2 Mean and SE (relative unit) of gene expression. Data are from expression in the undamaged and damaged leaf samples of both transgenic and control plants 95

G.1 Mean and SE of rust severity (on a categorical scale of 0 - 100) 98

H.1 Designation of transgenic and control lines 101

H.2 Screening for resistance toward *Septoria* leaf spot and stem canker in transgenic and control plants from clone 189-434 102

H.3 Screening for resistance toward *Septoria* leaf spot and stem canker in transgenic and control plants from clone 311-93 102

LIST OF APPENDIX TABLES (Continued)

Table | Page

H.4 Screening for resistance toward *Septoria* leaf spot and stem canker in transgenic and control plants from clone 195-529. 103

H.5 Clonal means for resistance toward *Septoria* leaf spot and stem canker. . 103

I.1 Mean lesion area developed in WS and WW trees over a 16-day period . 106

I.2 Lines ranking based on mean leaf damage following Kruskal-Wallis test. 106

J.1 Effect of copper ion concentration on histochemical *GUS* expression in CT (control) and FT (copper inducible) transgenics 109

J.2 Effect of copper ion concentration on histochemical *GUS* expression in two FT transgenic lines. 109

J.3 Histochemical *GUS* expression in whole plants induced with and without copper induction (50 μM $CuSO_4$) 110

J.4 Mean and SE of GUS activity (pmole 4-MU/min/mg protein) in two FT lines at different $CuSO_4$ concentrations, as determined by fluorometric assay. 111

LIST OF ABBREVIATIONS

ACE	=	Activating copper-metallothionein expression
ANOVA	=	Analysis of variance
bO	=	Bacterio-opsin
bp	=	Base pairs
CaMV	=	Cauliflower mosaic virus
cM	=	Centi-Morgan
CMV	=	Cucumber mosaic virus
CP	=	Coat protein
CT	=	Control construct
D	=	Damaged
DF	=	Degrees of freedom
DNA	=	Deoxyribonucleic acid
DDF	=	Denominator degrees of freedom
EST	=	Expressed-sequence tag
FT	=	Full construct
GLM	=	General linear model
GUS	=	β-glucuronidase
HLT	=	Healthy leaf tissue
HR	=	Hypersensitive response
kb	=	Kilo base pairs
ln	=	Natural logarithm
LSM	=	Least-squares means
Mb	=	Mega base pairs
MRE	=	Metallothionein transcription factor-binding site
MS	=	Murashige and Skoog medium
MT	=	Marchel Tract
MU	=	4-methyl umbelliferone
NDF	=	Nominator degrees of freedom
nos 3'	=	Nopaline synthase terminator
nptII	=	Neomycin phosphotransferase II
PA	=	Peavy Arboretum
PAL	=	Phenylalanine ammonia-lyase
PCD	=	Programmed cell death
PCR	=	Polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

PDR	=	Pathogen-derived resistance
pg	=	Picogram
PR	=	Pathogenesis-related
PVX	=	Potato virus X
QTL	=	Quantitative trait loci
R	=	Resistance gene
RAPD	=	Random amplified polymorphic DNA
RFLP	=	Restriction fragment length polymorphism
RNA	=	Ribonucleic acid
SA	=	Salicylic acid
SAR	=	Systemic acquired resistance
SE	=	Standard error
TLD	=	Total lesion density
TMV	=	Tobacco mosaic virus
TNV	=	Tobacco necrotic virus
UD	=	Undamaged
VI	=	Volume index
win	=	Wound-inducible
WS	=	Water-stressed
WW	=	Well-watered

PREFACE

This thesis is divided into four chapters followed by a bibliography and appendices. Literature review and background to the thesis are provided in Chapter 1. This project was started with the goal of enhancing disease resistance in poplar through genetic engineering. In tobacco, the *bO* gene had been shown to activate multiple defense mechanisms in the absence of a pathogen, and also caused heightened resistance to bacterial and viral pathogens in tobacco. Therefore, it was selected for evaluation in poplar. This work is presented in Chapter 2. Because the *bO* gene is driven by a constitutive promoter, had it elevated disease resistance, it is likely to have been necessary to regulate its expression to avoid deleterious effects on tree growth and adaptation. The ability to induce its expression, such as during disease epidemics, could be accomplished using dilute copper sprays. Thus, a copper-inducible expression system that was also found to be functional in tobacco was tested in poplar, and is presented in Chapter 3. Chapter 4 provides a summary of conclusions followed by a bibliography. The appendices comprise additional information supporting my findings and a catalogue of stored laboratory materials to facilitate future uses. In addition, two appendices describe in detail collaborative work carried out on our transgenic plants by the U.S. Forest Service. This will be co-published together with our own inoculation tests. All of the statistical analyses were performed using SAS software version 6.2 (SAS Institute Inc. 1990). Unless otherwise stated, values for mean and one standard error (SE) are presented in (Mean ± SE) format. Common Standard International (SI) units are used throughout the thesis.

GENETIC ENGINEERING OF DISEASE RESISTANCE IN POPLAR: EFFECTS OF BACTERIO-OPSIN OVER-EXPRESSION AND ANALYSIS OF A COPPER-BASED SYSTEM FOR RESISTANCE-GENE ACTIVATION

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

IMPACT OF DISEASES ON POPLAR PLANTATIONS

The genus *Populus,* which includes poplars, cottonwoods and aspens, contains 30 to 40 different species that are distributed widely over the Northern Hemisphere and subtropical regions (FAO 1985). Poplars are grown for many purposes, including: fuel, fiber, lumber, windbreaks, soil conservation, groundwater remediation, and rehabilitation of riparian areas. Because of their fast growth and easy propagation, poplars have been grown in plantations under short-rotations to provide a renewable source of biomass for energy, and to provide fiber for the pulp and paper industry (FAO 1997, Zsuffa et al. 1996). In the United States (U.S.), poplar has been successfully cultivated in plantations in the Pacific Northwest (PNW), North Central and Southern regions (Wright and Berg 1996).

Disease resistance is usually one of the major selection criteria in poplar breeding programs. Due to the diversity of pathogens in many poplar-growing areas, only a few clones display both desirable growth characteristics and adequate disease resistance. Nonetheless, resistance has usually broken down over time with co-evolution of pathogens (Newcombe 1996). Because poplar is easily propagated and cultivated worldwide, its pathogens also easily spread through introduction of plant materials into other regions.

Pathogens have caused significant losses in plantation productivity in many parts of the world (reviewed in Pinon 1984, Zsuffa et al. 1996). In the 1960's the Deuteromycetes pathogen, *Dothichiza populea*, became widespread and killed more than 100,000 trees throughout Germany within 10 years. This epidemic was the result of planting of a limited number of widely susceptible clones. Leaf-spot disease caused by *Marssonina brunnea* has led to substantial economic losses to euramerican poplars in Europe and *Populus deltoides* in the U.S. (FAO 1985). *Septoria* stem canker, which can reduce wood quality as well as cause mortality, is the primary cause of plantation failure when *Populus trichocarpa* hybrids trials have been planted in the midwestern U.S. (Ostry 1985). The disease can be serious in young plantations and may kill trees outright or reduce biomass yield by more than 60% in 3 years (FAO 1985, Ostry and McNabb 1985). This pathogen also has been very damaging to hybrid poplar plantations in North and South America, and has prevented planting of euramerican hybrids in Canada, the U.S. and Argentina (FAO 1985).

Rusts are the most important group of poplar diseases. In the U.S., *Melampsora* leaf rust can reduce volume growth by more than one-third (Widin and Schipper 1981) and kill young trees (Newcombe et al. 1994). In Australia and New Zealand, introduced leaf rust pathogens have caused substantial mortality in their exotic poplar plantings, leading to rapid turnover in the clones recommended for planting within just a few years (Pryor 1976). In the last decade, new races of *Melampsora larici-populina* Kleb. (races E2 and E3) have emerged in Europe (Pinon et al. 1987) and caused widespread plantation damage. None of the widely planted European cultivars are fully resistant to the new races, although some are resistant to an earlier race, E1 (Pinon and Valadon 1997, cited in Legionnet et al. 1999). One of the races of *M. larici-populina* was introduced into North America in 1991 and poses a major risk to American plantations (Newcombe and Chastagner 1993a). In Europe, *Venturia* leaf and shoot blight can cause up to a 30% loss of growth in susceptible clones (Giorcelli and Vietto 1992). In the Pacific Northwestern of U.S., many commercial clones, including many highly productive

P. trichocarpa x *P. deltoides* hybrids, have been replaced due to their susceptibility to *Venturia populina* (Newcombe 1996).

IMPORTANT DISEASES OF POPLAR

Melampsora leaf rust

One of the most important and well studied diseases of poplar is the leaf rust caused by the Basidiomycete fungus, *Melampsora* spp. It causes premature defoliation, resulting in growth reduction in old trees and mortality in young ones (Ostry et al. 1989, Newcombe 1996). *Melampsora* spp. are heteroecious, requiring two hosts to complete their life cycle: the aecial host is usually a conifer and the telial host is poplar. Infection starts in early spring during wet weather: over-wintering teliospores (2n) on poplar leaves infected in the previous season develop into basidiospores (n) which are then wind-blown to infect conifer hosts. Sexual recombination takes place in this alternate host and gives rise to aeciospores, which then infect poplar leaves in early summer. This is followed by the formation of uredia bearing urediniospores, which can be seen as orange pustules on the undersides of leaves during mid to late summer. The spores continue to attack poplar leaves repeatedly by producing more uredia and urediniospores until they finally reach the over-wintering stage at the end of the season (Agrios 1988, Ostry et al. 1989).

Melampsora spp. are very host-specific (reviewed in Newcombe 1996). In North America, *M. occidentalis* H. Jack attacks the western black cottonwood (*P. trichocarpa* Torr & Gray) while *M. medusae* Thuem. is pathogenic to *P. deltoides* Bartr. Ex. Marsh. and *P. tremuloides* Michx., but not to *P. trichocarpa*. However, these rust species have recently expanded beyond their native geographical regions and are colonizing new poplar species. *Melampsora occidentalis* was observed in the Midwest in the early 1990s (Moltzan et al. 1993), and *M. medusae* f. sp.

deltoidae, which is native to the eastern cottonwood (*P. deltoides*), was first observed in hybrid plantations in the PNW in 1991 (Newcombe and Chastagner 1993b). Intercontinental spread has also occurred; the notorious rust of Europe, *M. larici-populina* Kleb., was first observed in North America in 1991 (Newcombe and Chastagner 1993a).

Poplar resistance to *Melampsora* rust is under strong genetic control, and often involves major genes that provide resistance to specific fungal races (Thielges and Adams 1975). For example, a major gene for resistance to *M. medusae* f. sp. *deltoidae* (*Mmd1*) governs necrotic flecking, a form of hypersensitive response. The single dominant gene was provided by the *P. trichocarpa* parent in a hybrid poplar pedigree (reviewed in Newcombe 1996, Bradshaw 1996). Other major genes include those for resistance to different races of *M. larici-populina* (Cervera et al. 1996, Villar et al. 1996); a cluster of genes with multiple alleles that confer resistance to two races of *M. larici-populina* E1 and E3, and two additional tightly linked loci that confer resistance to race E2 (Lefevre et al. 1998). These major genes are also "assisted" by minor genes or quantitative trait loci (QTL) for resistance (Newcombe 1998). Two QTLs for resistance to rust have been mapped (Newcombe 1996).

Venturia leaf and shoot blight

Venturia spp. cause leaf and shoot blight of poplars worldwide while their relatives cause the well-known disease of apple, apple scab. *Venturia* spp. are sexual members of the sac fungi, Ascomycete. Their asexual stage *Pollacia*, belongs to the imperfect fungi group (Deuteromycete) (Agrios 1988). The life cycle requires a primary infection in early spring when pseudothecia, which have over-wintered on blighted shoots and leaves, ejaculate ascospores that infect newly expanding leaves. The disease spreads through midveins and forms brown to black necrotic lesions on young leaves, causing a distinctive curl at the leaf margins.

Petioles and shoots also exhibit necrosis. The shoot tissues are black, brittle and curled to resemble a "shepherd's crook," the defining featuring of *Venturia* infection in *Populus*. Overlying the necrotic lesions is a conidia-bearing layer, which has a velvety texture when touched. Conidia can produce secondary infections causing rapid disease amplification (Agrios 1988).

Two species that cause serious levels of leaf and shoot blight in commercial poplar plantings are *V. populina* Vuill. Fabr. and *V. tremulae* Ader. (Ostry et al. 1989). Their anamorph (conidial) states are known as *P. elegans* Serv. and *P. radiosa* (Lib.) Bald. & Cif., respectively (Dance 1961). The specificity of *Venturia* spp. on the poplar host is restricted to the taxonomic section level of *Populus* (reviewed in Newcombe 1996). Both species are widespread in Europe and North America. *Venturia populina* causes disease in the sections *Tacamahaca* Spach and *Aigeiros* Duby in Europe and India, and only to *Tacamahaca* in North America. *V. tremulae* causes disease exclusively on the section *Populus* in both Europe and North America.

In the PNW, *V. populina* infects black cottonwood, balsam poplar (*P. balsamifera* L.) and hybrids of *P. trichocarpa* x *P. deltoides*. Many productive clones from early breeding programs in North America that were widely planted succumbed to *V. populina* (Newcombe and Oosten 1997), resulting in their removal from planting programs. Host resistance to *V. populina* is under strong genetic control; hybrids of *P. trichocarpa* x *P. deltoides* vary from highly resistant to highly susceptible, hybrids of *P. trichocarpa* x *P. nigra* L. vary from moderately to highly resistant, and hybrids of *P. trichocarpa* x *P. maximowiczii* Henry are highly resistant (Newcombe and Oosten 1997).

***Septoria* leaf spot and stem canker**

Leaf spot and stem canker caused by *Septoria* spp. can cause mortality and reduce yield in young plantations. In poplar, two species are responsible for the

disease. *Septoria musiva* Peck causes both diseases, while *S. populicola* Peck causes only leaf spot (Ostry et al. 1989). *Septoria* spp. belong to the imperfect fungi (Deuteromycete). Infection begins in spring when wind and rain carry ascospores from perithecia on diseased leaves and infected stems to new leaves, branches and stems. Leaf spots begin as small yellowish specks, which later enlarge and turn brown or black with brown or yellow margins. Clusters of black pycnidia are predominant in the center of the spots, and bear conidia for secondary infection. Infection on branches and stems leads to the formation of rough, ash-gray areas with cracks. These cankers can weaken stems and branches, making them prone to breakage and mortality (Agrios 1988, Ostry et al. 1989).

Poplar clones differ in their resistance to *Septoria* (reviewed in Newcombe 1996). *Populus trichocarpa*, which is the native black cottonwood of the PNW, is susceptible to the leaf spot and stem canker diseases caused by *S. populicola* and *S. musiva*, respectively. However, only *S. musiva* has been detected in the eastern U.S., and it has severely affected *P. trichocarpa* research plantations there. Meanwhile, *P. deltoides*, the eastern cottonwood, is resistant to *S. populicola* in the PNW, but is susceptible to *S. musiva*. Three dominant QTLs conferring resistance to *S. populicola* have been mapped (Newcombe and Bradshaw 1996).

DISEASE RESISTANCE

Plants are vulnerable to attack from diverse microorganisms and insects. Plant defense mechanisms rely on both preformed and inducible defenses (Agrios 1988, Greenberg 1997). Under pathogen invasion, defense mechanisms are activated both locally and systemically. Plant-pathogen interaction is said to be incompatible (host resistant, pathogen avirulent) when a pathogen is unable to overcome plant defenses. When invasion is accomplished and disease occurs, the interaction is known as compatible (host susceptible, pathogen virulent) (Staskawicz et al. 1995). Inducible forms of defenses have received a great deal of

attention because they can be readily directed towards engineering broad-spectrum resistance in transgenic plants (see below).

Many kinds of transgenic plants expressing foreign genes that can facilitate pest control via resistance to herbicides, insects and pathogens have been produced. Land usage for planting transgenic crops has increased in U.S. from 3.2 million hectares in 1996 to over 20 million hectares in 1998 (APHIS website; http://www.aphis.usda.gov). In the U.S., more than 30 genetically modified (GM) crops with various kinds of agronomic traits have been approved for commercial production and many more are under trial (Williams 1998, APHIS website; http://www.aphis.usda.gov/bbep/bp/images /status11.gif). Only three out of the 30 GM crops are disease-resistant plants: all are resistant to various viruses (listed below). The induced resistance was mediated by viral coat proteins (CP), the primary kind of transgenic disease resistance in commercial production (reviewed in Dempsey et al. 1998).

Natural mechanisms of disease resistance

Preformed defenses against pathogens include structures which act as physical barriers to inhibit entrance and progression, and antimicrobial chemicals (Agrios 1988). Induced mechanisms include diverse cellular changes to create an environment inhibitory to pathogen growth, and outright cell death via the hypersensitive response (HR). Hypersensitive response, a form of programmed cell death (PCD), is manifested in the form of necrotic lesions at the site of infection. It produces a toxic environment that restricts growth and spread of the pathogen (Dixon and Harrison 1992).

Hypersensitive response-induced cell death can occur during both incompatible and compatible interactions (Greenberg 1997). The nature of a pathogen determines how host cell death affects pathogenesis. Because obligate parasites (biotrophs) rely on living cells to grow, cell death would deprive these

pathogens of food. Saprophytic parasites (necrotrophs), however, may benefit from nutrients released by dead cells (Greenberg 1997). Because HR is accompanied by other general defense functions (reviewed in Hammond-Kosack and Jones 1996, Greenberg 1997, Gilchrist 1998), it also usually deters the growth of necrotrophic pathogens. Common HR-associated responses include fortification of cell walls, generation of an oxidative burst, and production of antimicrobial compounds including phytoalexins. Several pathogenesis-related (PR) proteins are also synthesized, including chitinase (*PR-3*) and β-1,3-glucanase (*PR-2*) that hydrolyze fungal cell walls. The localized high-level expression of PR proteins and accumulation of salicylic acid (SA) are often correlated with the activation of systemic acquired resistance (SAR). Systemic acquired resistance develops hours to days after infection, and provides prolonged, broad-spectrum protection throughout the plant (Ryals et al. 1996).

Genetic engineering of disease resistance

There are many approaches to genetic engineering of disease-resistance in plants (reviewed in Shah 1997, Bushnell et al. 1998, Dempsey et al. 1998). Because diseases are caused by diverse kinds of microorganisms with distinct means of pathogenesis, the nature of each plant-pathogen interaction has to be taken into consideration when engineering resistance. Genetic engineering of viral resistance has been more extensively studied than bacterial or fungal resistance, and has been very fruitful. In developing virus-resistant transgenic plants, the genes or sequences derived from viral genome have been transformed into susceptible plants (reviewed in Baulcombe 1996, Ratcliff et al. 1997). There are two mechanisms by which this resistance, termed pathogen-derived resistance (PDR), is mediated. One is protein-mediated PDR, which seeks to provide broad protection. The second is RNA-mediated PDR, which is viral-specific but confers a very high level of resistance. The first type of resistance requires expression of virus-derived protein,

while the second type induces RNA-mediated gene silencing. Gene silencing causes degradation of viral RNA required for pathogenesis (reviewed by Bruening 1998, Waterhouse et al. 1998). The first example of protein-mediated PDR came from transgenic tobacco expressing the CP gene of tobacco mosaic virus (TMV). Coat protein-mediated PDR has successfully enhanced resistance to viruses in several plant species (Baulcombe 1996). A few CP-transgenic crops are now commercially available, including: transgenic squash plants that are resistant to watermelon mosaic virus 2 (WMV2) and zucchini yellow mosaic virus (ZYMV); other transgenic squash plants that are resistant to cucumber mosaic virus (CMV) in addition to WMV2 and ZYMV; and transgenic papaya plants which resist papaya ringspot virus (PRSV) (APHIS website; http://www.aphis.usda.gov/bbep/bp/not_reg.html).

Resistance against fungal and bacterial diseases has been engineered by expression of antimicrobial compounds (reviewed in Shah 1997, Bushnell et al. 1998, Dempsey et al. 1998). Among the antimicrobial genes utilized are those encoding for phytoalexins, which are small, broad-spectrum antimicrobial compounds. Other tools include various PR proteins, which are induced in response to pathogenesis. They include chitinases and β-1,3-glucanases, both of which possess *in vitro* antifungal activity. These enzymes hydrolyze chitin and glucan, which constitute the polymeric backbone of fungal cell walls. They are also associated with the HR and strongly expressed during incompatible host-pathogen interactions. However, constitutive expressions of single genes, or the introduction of multiple PR genes, have varying effectiveness at conferring resistance (Broglie and Broglie 1993, Shah et al. 1995). A number of studies (reviewed below) reported that partial resistance was conferred by the PR genes when transgenic plants were challenged in laboratory or greenhouse. Broglie et al. (1991) introduced a chitinase gene from bean into tobacco and canola plants. The transgenics exhibited increased but incomplete resistance to infection by *Rhizoctonia solani*. In contrast, Neuhaus et al. (1991) found that a chitinase gene expressed in transgenic tobacco plants failed to provide measurable resistance to

Cercospora nicotianae infection. In other studies, transgenic tobacco plants expressing chitinase and β-1,3-glucanase, either singly or combinatory, were more resistant to *R. solani* (Jach et al. 1995). Recently, plants expressing an endochitinase gene isolated from a biocontrol fungus *Trichoderma harzianum* were found to be highly resistant to various foliar and soil-borne pathogens during greenhouse and laboratory experiments. This is the first report of using such novel genes for engineering disease resistance (Lorito et al. 1998). However, despite many laboratory studies, there are no cases of commercial transgenic crops using this strategy (APHIS website; http://www.aphis.usda.gov/bbep/bp/not_reg. html) suggesting that strong, stable resistance may be hard to deliver by this approach.

Some other antifungal proteins that may induce resistance are thionins and defensins, which are present in vacuoles and cell walls of higher plants, especially in seeds. These are small antifungal peptides, though sometimes they also have antibacterial activity (reviewed in Broekaert et al. 1997). Their antimicrobial activities have been demonstrated *in vitro*; however, their modes of action are poorly understood (Dempsey et al. 1998). Through laboratory assays, the over-expression of an *Arabidopsis* thionin gene has been shown to elevate resistance against *Fusarium oxysporum*, and expression of a defensin gene (*Rs-AFP2*) from radish seed in transgenic tobacco plants enhanced resistance to *Alternaria longipes*. The expression of two defensin genes, *AX1* and *AX2*, from sugar beet leaves in transgenic corn plants gave promising results for resistance to Northern corn leaf blight caused by the foliar pathogen *Exserohilium turcicum* in laboratory assays (reviewed in Shah 1997, Dempsey et al. 1998).

Most strategies for genetic engineering of resistance against bacterial pathogens are designed to lyse bacterial cells or prevent bacterial growth. For example, expression of a lysozyme gene from bacteriophage T4 in transgenic potato successfully enhanced resistance to *Erwinia carotovora* in both laboratory and greenhouse experiments (During et al. 1993, During 1996). Other methods use genes encoding for cecropins, bacterial peptides that disrupt bacterial membranes,

plant thionins, bacterial toxins, and various compounds that contain antibacterial properties (During 1996).

Recently, many plant resistance genes (R) that provide Mendelian, major-gene resistance to plant pathogens have been cloned (reviewed in Hammond-Kosack and Jones 1997, Parker and Coleman 1997). Some of these genes, such as *Xa21*, *Bs2* and *Mlo*, are able to confer resistance to multiple pathogen races. Several of these classes of genes are involved in disease perception and signal transduction. These include the tomato *Prf*, *Pti1*, and *Pti4-6* genes (reviewed in Shah 1997, Demsey et al. 1998, Higgins et al. 1998). Michelmore (1995) proposed a strategy that involves introducing multiple R genes (*i.e.* pyramiding) to create multigenic resistance against all races of a pathogen through direct transformation into susceptible plants. Alternatively, de Wit (1992) proposed a strategy to use a form of "gene cassette" via transformation: a two-component system consisting of the avirulence (avr) gene and its corresponding R gene. This is an example of manipulating the gene-for-gene interaction, where a certain avirulence gene product from a pathogen is recognized by a complimenting R gene in the host, thus eliciting HR. One example is the *Avr9-Cf9* two-component system; these genes were derived from tomato and its pathogen *Cladosporium fulvum* (de Wit 1992, reviewed in de Wit 1997). Although this approach has a potential to produce broad-spectrum protection, it requires a promoter that acts specifically upon pathogen invasion.

Genetic engineering of HR-based resistance

During HR, a signal transduction pathway is triggered upon recognition of the invading pathogen, resulting in the orchestrated activation of various plant defense mechanisms (reviewed in Greenberg 1997, Hammond-Kosack 1997). Other components include the accumulation of SA, production of PR proteins, induction of antimicrobial compounds, heightened expression of *PAL*, and rapid

cell death at the site of infection (reviewed in Mittler and Lam 1996, del Pozo and Lam 1998). In both animals and plants, a genetically regulated cell death pathway is known to play a role in developmental processes, and referred to as 'apoptosis' or programmed cell death (PCD) (reviewed in Gilchrist 1998). A variety of plant mutants termed "disease lesion mimics" display spontaneous lesions similar to HR lesions, but in the absence of a pathogen, demonstrating the existence of genes regulating PCD (Dangl et al. 1996, Mittler and Lam 1996).

Several transgenes have been used to create transgenic plants expressing lesion mimic phenotypes, including the *bO* gene, the yeast invertase gene and the cholera toxin gene (reviewed in Shah 1997). In tobacco and potato plants expressing *bO*, several biochemical markers associated with the onset of HR were elevated in the absence of pathogens. None of the plants showed any growth or developmental abnormality (Mittler et al. 1995, Abad et al. 1997). A similar result was observed in *Arabidopsis* (Lam E., pers. comm.). Interestingly, both tobacco and potato *bO*-transgenic plants exhibited heightened resistance to certain viral and bacterial pathogens, in laboratory assays or growth chamber trials. Transgenic tobacco plants demonstrated enhanced resistance to TMV, tobacco necrotic virus (TNV) and *Pseudomonas syringae* pv *tabaci* in laboratory assays. In addition, *bO*-transgenic potatoes were also resistant to the US1 isolate of the fungal pathogen *Phytophthora infestans*, but not to the US8 isolate, in growth chamber trials. However, *bO* over-expression in potatoes failed to provide resistance to the bacterial pathogen *E. carotovora*, and actually increased susceptibility to PVX in laboratory experiments (Abad et al. 1997). Hence, it appears that the *bO*-induced resistance is both pathogen and host specific.

The *bO* gene was isolated from a bacteriorhodopsin (*Halobacterium halobium*), which encodes a protein that functions as a light-driven proton pump to increase the photosynthetic capacity of higher plants (Krebs and Khorana 1993). The mechanism by which *bO* gene induces lesions is unknown. However, because it functions as a proton pump, it was proposed that the artificial increase in proton translocation activity across membranes might alter cellular homeostasis and be

misinterpreted by the host cell as pathogen invasion, thus triggering HR. Alternatively, the unbalanced biochemical state may mimic an early event in the elicitation of defense response leading to activation of the cell death pathway (Mittler et al. 1995).

GENETIC ENGINEERING OF POPLAR

Poplars have a number of advantages as model systems for tree biotechnology (Bradshaw 1998). They are very fast-growing, can be easily propagated vegetatively, have a small genome, and are amenable to genetic transformation. Genome maps exist in a number of pedigrees and thousands of transgenic trees have been created. A large expressed-sequence tag (EST) project is underway that is expected to generate tens of thousands of partial cDNA sequences (Sterky et al. 1998).

Molecular genetics

Poplar genetics has been studied extensively, with most effort directed towards heterosis, adaptation, physiology and disease resistance (Bradshaw and Stettler 1993, Bradshaw et al. 1994, Bradshaw and Stettler 1995). Poplars are predominantly diploids (2n=38) with a small genome (500 Mb haploid), about five times bigger than *Arabidopsis* and similar in size to rice. The ratio between physical and genetic length is approximately 200 kb/cM, comparable to that of *Arabidopsis*.

A three-generation *P. trichocarpa* x *P. deltoides* hybrid pedigree developed at the University of Washington is widely used by researchers for genome mapping and studies of trait inheritance. Using this pedigree, a dense genetic map has been constructed using RFLP/RAPD markers and QTLs related to growth traits (Bradshaw et al. 1994, Bradshaw and Stettler 1995). In addition, three QTLs

governing resistance to *S. populicola* and two loci for resistance to rust have been located (Newcombe 1996, Newcombe and Bradshaw 1996).

Agrobacterium-mediated transformation

A transformation system mediated by *Agrobacterium* has been well established in poplar making genetic engineering feasible (reviewed in Han et al. 1996, Han et al. 1999). Diverse genotypes can now be routinely transformed and regenerated into plants. Through genetic engineering, several desired traits have been incorporated into poplars. Transgenic hybrid poplars tolerant to herbicides and resistant to insects have been produced and their performance in the field is currently being studied (TGERC 1998).

Transformation is an important tool for testing gene function. With identification of disease resistance loci on the poplar genetic map, it should be possible to clone major resistance genes (or other Mendelian genes of interest) through positional cloning. Alternatively, candidate resistance genes might be cloned from poplar by using a polymerase chain reaction (PCR) approach. Amino acid sequence comparisons among a number of disease resistance genes cloned from diverse species possess conserved motifs (reviewed in Hammond-Kosack and Jones 1997). This has enabled resistance gene homologs to be directly isolated from diverse species using degenerate primers (Michelmore 1996). In the future, these cloned resistance genes can be used as probes to both study linkage to disease resistance traits and to isolate entire resistance loci for use in transformation.

REGULATING TRANSGENE EXPRESSION

Transgene expression is primarily controlled by DNA sequences 5' to the translation start site, usually referred to as the promoter (Alberts et al. 1994). Currently, most genetically modified plants contain herbicidal, insecticidal or viral

resistance genes. Because broad and strong expression of these genes is usually desired, a constitutive promoter such as the 35S promoter from cauliflower mosaic virus RNA (CaMV 35S) is often used. However, for many other kinds of transgenes, expression throughout plant development is not desirable, and may cause abnormal growth. For example, many kinds of disease resistance genes are normally expressed only after pathogen attack, and would be likely to have deleterious effects if over-expressed continuously. A number of different systems for control of transgene expression have been tested, the most common being the 'chemical-inducible gene expression systems'.

Inducible promoter systems

Some promoters can be regulated through the presence or absence of specific chemical inducers. An ideal inducer would give a very low level or no expression in the absence of the inducer and a very high level of expression in the presence of the inducer. Additionally, the inducer should be specific, having no effect on other cell functions or on the expression of other genes. Discussion of the characteristics of good inducers, and their suitability for genetic engineering, can be found in Gatz and Lenk (1998).

Several chemical-inducible promoters have been studied (reviewed in Gatz and Lenk 1998, Jepson et al. 1998), both of plant and non-plant origin. An example of an endogenous inducible plant promoter is the *PR-1a* promoter from tobacco, which drives expression of a defense gene, naturally induced by pathogen invasion, and can also be induced by chemicals such as SA, benzothiadiazole (BTH) and isonicotinic acid (INA). Transgenic plants have been produced that contain the *Bacillus thuringiensis* δ-endotoxin gene under the control of the *PR-1a* promoter (Gorlach et al. 1996); INA treatment led to insect resistance. Alternatively, two safener-inducible promoters derived from maize, *In2-2* and *GST-27*, can be used under the influence of safener benzenesulfonomide, an

agrochemical that can reduce toxic effects of certain herbicides on plants (de Veylder et al. 1997). Transgenic *Arabidopsis* plants containing the *In2-2* promoter fused to a reporter gene have successfully directed expression in specific tissues. When safener is present, reporter activity was detected in the roots, apical meristems and hydathodes (de Veylder et al. 1997).

Several regulatory genes from prokaryotes that respond to chemicals have been identified (reviewed in Gatz and Lenk 1998, Jepson et al. 1998). However, the strategy for using these promoters is more complex than endogenous plant promoters because they usually require the expression of two genes; a gene encoding a regulatory protein and a gene of interest under control of a suitable promoter. Two-component expression systems include those that respond to tetracycline, dexamethasone, copper, ethanol, and lactose (reviewed in Jepson et al. 1998). An inducible system that works well in one plant does not always work adequately in others. Because different kinds of plants have different physiology, the effectiveness of a gene induction system varies significantly. For an example, the tetracycline induction system provides effective timing control for transgene expression in tobacco cells. However, its spatial control throughout whole tobacco plants is variable, and the system appears to be completely ineffective in *Arabidopsis* (Gatz 1997).

Copper-inducible gene expression system

A gene expression system responsive to external applications of copper II ion was demonstrated in tobacco and *Lotus* (Mett et al. 1993, Mett et al. 1996). The system was based on the yeast copper-metallothionein regulatory system. It involves two components in a reporter-gene version (Figure 1.1). The β-glucuronidase (*GUS*) reporter gene is driven by a 90-bp domain A of the CaMV 35S promoter (CaMV 35S -90) attached to the *ACE1* transcription factor-binding site (MRE), and the *ACE1* (activating copper-metallothionein expression)

regulatory gene is under control of CaMV 35S. In the presence of copper ions, ACE1-copper complex binds to the MRE element in the hybrid promoter (MRE::CaMV 35S -90) to activate *GUS* transcription.

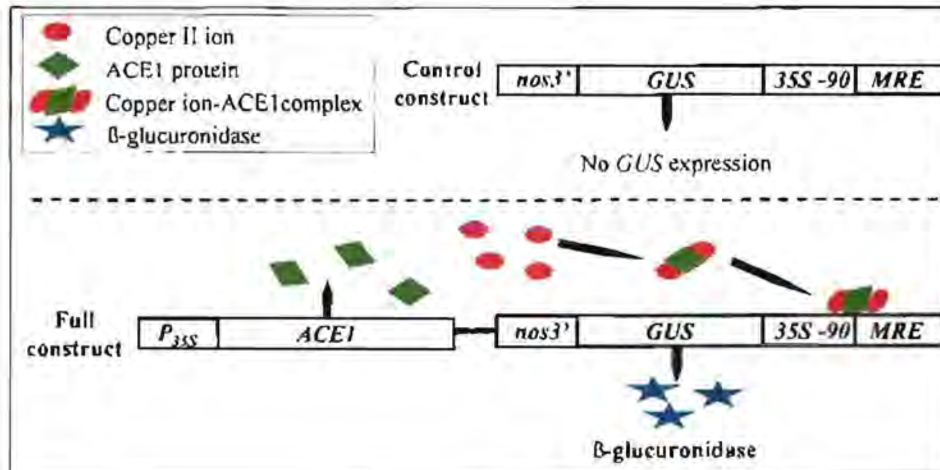

Figure 1.1 The idealized mechanism of action of a copper-inducible gene expression system (after Mett et al. 1993). In the presence of copper II ion (lower panel), the ACE1 protein binds copper and associates with the transcription factor-binding site (MRE), stimulating transcription. In the absence of copper II (upper panel), ACE1 is unable to effectively bind the MRE element, preventing *GUS* expression. P_{35S} = CaMV 35S promoter; *nos3'* = polyadenylation terminator signal; *35S-90* = 90-bp domain A of the CaMV 35S.

CHAPTER 2

EFFECT OF OVER-EXPRESSION OF A BACTERIO-OPSIN GENE ON LESION FORMATION, GROWTH AND DISEASE RESISTANCE IN HYBRID POPLAR

ABSTRACT

We studied the ability of the *bO* gene to promote disease resistance and expression of defense-related genes in transgenic poplars (*Populus*). Over-expression of *bO* had previously been shown to induce HR-like lesions, increase viral and bacterial disease resistance, and stimulate PR gene expression in tobacco. We generated 35 transgenic lines of two clones of *P. trichocarpa* x *P. deltoides* hybrids (clone 189-434 and 195-529) and one clone of a *P. trichocarpa* x *P. nigra* hybrid (clone 311-93), and challenged them with the fungal pathogens *Melampsora* (rust), *Venturia* (leaf and shoot blight), and *Septoria* (leaf and stem canker) in greenhouse, field and laboratory inoculations. Northern analysis and lesion development showed that the *bO* gene was expressed in the transgenic poplars, however, no increase in resistance or in RNA expression of *PAL1*, *win6* or *win8* was observed, even in a line that showed very high *bO* expression, rapid lesion development, and retarded growth.

INTRODUCTION

Plants have developed complex defensive mechanisms as a result of their long history of co-evolution with pathogens. In the course of infection, a pathogen has to successfully overcome the preexisting physical and chemical barriers in plants, and cope with systems programmed to recognize pathogens and induce new defense mechanisms. A primary induced mechanism of resistance by plants is the

hypersensitive response (HR). It is characterized by the formation of necrotic lesions resulting from localized cell death at the site of infection. The HR restricts further growth and spread of the pathogen into neighboring healthy cells (Dixon and Harrison 1992, Staskawicz et al. 1995), thus appears to be a programmed suicidal act of plant cells (Greenberg 1997). Concurrent with the onset of the HR is the activation of overall defense responses of plants (Hammond-Kosack and Jones 1996, Greenberg 1997, Gilchrist 1998). These include reinforcement of the plant cell wall, generation of an oxidative burst, accumulation of phytoalexins, and activation of defense-related genes. In addition, accumulation of salicylic acid (SA), a chemical compound associated with the activation of systemic acquired resistance (SAR), is also observed. The SAR induces prolonged and broad-spectrum protection throughout plants (Ryals et al. 1996).

Some of these "induced" defense mechanisms have also been observed in plants in the absence of pathogens. "Disease lesion mimics" that display HR-like lesions have been identified in diverse plant species (Dangl et al. 1996). Evidence from lesion mutants of *Arabidopsis* shows that they exhibit heightened resistance to bacterial and fungal pathogens (Dietrich et al. 1994). In addition, several transgenes have been used to create transgenic plants that express lesion-mimic phenotypes. These include the bacterio-opsin (*bO*) gene, the yeast invertase gene, and the cholera toxin gene (Shah 1997).

Tobacco plants expressing the *bO* gene from *Halobacterium halobium* with a HR-lesion phenotype were first reported by Mittler et al. (1995). They also reported that expression of several defense-related genes and SA, which are associated with the onset of HR, were elevated in *bO*-transgenic plants in the absence of pathogen attack. The transgenics also had heightened protection against two viral pathogens, tobacco mosaic virus (TMV) and tobacco necrotic virus (TNV), and a bacterial pathogen (*Pseudomonas syringae* pv *tabaci*) when the plants were challenged and analyzed through laboratory assays. Similar results of up-regulated pathogenesis-related (PR) genes and SA in transgenic potato expressing *bO* were obtained recently by Abad et al. (1997). However, protection

against pathogens was not very successful when the *bO*-potatoes were challenged with several pathogens in growth chamber trials and through laboratory assays. The *bO* gene enhanced resistance toward one race of a fungal pathogen (*Phytophthora infestans* mating type A1) but failed to impart resistance toward another race of the same pathogen (mating type A2). It also failed to impart resistance to a bacterial pathogen (*Erwinia carotovora*), and it actually increased susceptibility to a viral pathogen (potato virus X, PVX) (Abad et al. 1997). Contrary to expectations that promotion of the HR via the *bO* gene could provide broad-spectrum resistance, instead it appears that *bO*-induced resistance has specificity (Abad et al. 1997).

To test whether the *bO* gene would stimulate the HR response and increase disease resistance in poplar, we produced transgenic trees using clones that are susceptible to different types of fungal pathogens that cause major disease epidemics in poplar plantations (Newcombe 1996). We report that although over-expression of *bO* led to the generation of lesion-mimic phenotypes in hybrid poplar leaves, it did not elevate expression of defense-related genes nor affect pathogen resistance in the lab, greenhouse, or field tests.

Materials and Methods

Bacterial plasmids and strains

The vector pEL301 (Mettler et al. 1995) was provided by Dr. Eric Lam (Rutgers University). This binary vector's T-DNA contains a synthetic *bO* gene (Nassal et al. 1987), a chlorophyll a/b transit peptide (*Cab-t*), and an Ω translation-enhancing sequence. Expression of the whole cassette (Ω-*Cab-t-bO*) was driven by the CaMV 35S promoter and the nopaline synthase terminator (*nos 3'*). This transcriptional unit was inserted into pBI100 T-DNA binary vector (Clontech, CA), which has a kanamycin resistance gene (*nptII*) for *in vitro* selection, giving rise to

pEL301. Plasmid EL301 was transformed into competent *Escherichia coli* DH5α (Gibco BRL) for multiplication. It was then transformed into *Agrobacterium tumefaciens* strain C58 for poplar transformation.

Plant materials and transformation

Plasmid EL301 was transformed into three hybrid cottonwood clones: clone 189-434 and 195-529 (*P. trichocarpa* x *P. deltoides*), and clone 311-93 (*P. trichocarpa* x *P. nigra*) following an established *Agrobacterium*-mediated transformation protocol (Han et al. 1999). Transgenic plants were selected based on their ability to root in the presence of kanamycin (25 mg/L), via detection of the *bO* transgene based on PCR (Appendix A), and via northern analysis of *bO* transgene expression. Total genomic DNA was isolated from individual transformants using a small-scale DNA preparation protocol (http://www.fsl.orst. edu /tgerc/dnaext.htm). After micropropagation *in vitro*, plants were potted in soil and grown in the greenhouse for 5-6 months before being planted in the field for analysis of both growth and resistance to *Venturia*. To study rust resistance, 19 selected lines were grown in 5 x 5 cm pots in the greenhouse until the age of 3-4 months. Two ramets of each line and controls were placed randomly in a tray and then inoculated. For the *Septoria* assays, three ramets each of 36 transgenic lines and 3 control plants were grown in the greenhouse until the age of 2-5 months, then leaf and stem tissues were used in *in vitro* inoculations.

Field trial and spontaneous lesion assessment

To test their performance in an uncontrolled environment, transgenic poplars were transplanted into two different field sites near Corvallis, Oregon, U.S. (latitude= 44° 34', longitude= 123° 16'), in summer 1997. Three ramets of each of 38 independent transformants (lines) and non-transgenic controls (3 clones) were

planted in a completely randomized design. The two sites were at Peavy Arboretum (PA) and Marchel Tract (MT). These sites differed in several aspects. The PA site is in a hilly area, has a hard clay soil, and Douglas-fir trees are abundant in the vicinity. The MT site has a sandy soil, is in the flood plain of a large river (Willamette), and is near to extensive wild and planted poplars. Trees were planted on a 14.6 x 23.2 m plot with a spacing of 1.8 m between rows and 1.2 m in between trees within a row (18 trees per row; 7 rows). To study how the *bO* transgene might affect tree growth, height and diameter were measured two weeks after planting, and again in fall 1998, and volume index was used to assess yield. Tree volume index (VI) was calculated as height x diameter^2 (cm^3), and data were transformed into natural logarithm (ln). Net growth was the difference between the ln of VI at the beginning and the ln of VI2 at the end of a growing season (ln VI2 – ln VI1). This was done to account for differential plant size at the time of planting and their non-linear growth rates. Growth data was analyzed via SAS (version 6.2, SAS Institute Inc. 1990) using general linear models (GLM) and analysis of variance (ANOVA) procedures.

Spontaneous lesions (necrotic tissues) were scored on field-grown trees in early spring 1998. We identified the first fully opened leaf on the main stem as leaf number 1, and the six leaves below it as leaf numbers 2 to 7. Lesions began to form and were most readily recognized on these leaves. Lesions severity was rated by counting the number of lesions that developed on leaves 2 to 7 and categorized as: 0=no lesions, 1=1-5 lesions, 2=6-10 lesions, and 3= >10 lesions (or too many to count). Because lesions were irregular in shape and size, the overall proportion of necrotized tissue (brown spots) on each leaf was also rated as: 1=1-25%, 2=26-50%, 3=51-75% and 4=76-100% and were given weights of 5, 15, 23 and 33.3 (similar to scoring *Venturia* symptoms, described below). Multiplication of the severity and necrosis indices gave an estimate of lesion area per leaf. Because leaves 2 to 7 varied in size, we also estimated the area for each indexed leaf. Leaves were gathered from 6 transgenic lines and the three non-transgenic clones, and leaf area was measured using a grid on leaves 2 to 7. Lesion density (in

relative units) was determined for every indexed leaf by dividing mean lesion area by mean leaf area for each clone. By adding lesion density values for leaves 2 to 7, we obtained the total lesion density (TLD) for each ramet.

Fungal inoculation and disease assessment

Transgenic plants were screened for resistance to four different fungal pathogens. *Melampsora occidentalis* rust urediniospores were collected from diseased leaves in early fall 1997, from a plantation near Monmouth, Oregon (35 km north of Corvallis). Conidia of *Venturia populicola* leaf and shoot blight fungus were collected in mid-spring 1998 from newly infected leaves of hybrid poplar in a different plantation, also near Monmouth, Oregon. They were used directly for inoculation in the greenhouse and field. *Septoria musiva* and *S. populicola* cultures were obtained from leaves of hybrid poplar clone NE 299, near Milaca, Minnesota, and from black cottonwood leaves near Toutle, Washington, respectively. Cultures were maintained on V-8 juice agar at 20 ^{o}C under continuous light.

For rust inoculation, we prepared a urediniospore suspension at a concentration of 3 x 10^4 spores/ml in 0.05% Tween 20 (Newcombe et al. 1996). Plants were inoculated individually by brushing each leaf with the spore suspension. We selected a total of 19 transgenic lines drawn approximately evenly from three clones (189-434, 195-529 and 311-93), as well as the three non-transgenic control clones. Experiments utilized two 3- to 4-month old ramets from each transgenic and control line in a completely randomized design (in a tray), and were repeated twice in time (September – November 1997). Two batches of plants were prepared, one was inoculated with rust, and the other was brushed with sterile water, to serve as an inoculation control. After inoculation the trays were transferred into a plastic tent (1.8 x 1.5 x 1.1 m) on the greenhouse bench and sealed to keep leaves moist; control trays were placed under a separate tent. After

18 h, the trays were taken out and placed on the bench at a constant greenhouse temperature of about 20 ^{0}C. Disease severity was assessed 14 days later following the method of Schreiner (Schreiner 1959, Newcombe et al. 1996). A rating of 1 (light), 5 (moderate) or 25 (heavy) was given to the most severely rusted leaves on an individual plant basis. Occurrence of rust was given a value of 1, 2, 3 or 4, by estimating the overall proportion of rusted leaves on each plant in classes: $\leq$ 25, 26-50, 51-75, or >75%. Multiplication of the rating and occurrence values gave a Schreiner rating that ranged from 0 (highly resistant) to 100 (highly susceptible).

A conidial suspension was prepared at a concentration of 5 x 10^4 conidia/ml by brushing the black, velvety lesions from freshly *Venturia* infected leaves into distilled water (Newcombe and Oosten 1997). Nine-month old trees grown in the field were sprayed with the suspension, which was allowed to dry for 2 h. Then, to keep leaves moist, an individual plastic tent was put over each tree using a clear plastic bag (approximately 61 x 91 cm) mounted on a conical bamboo frame. After 48 h, the tent was removed; disease severity was assessed after 2 weeks. Leaf and shoot blight symptoms were rated following Newcombe and Oosten (1997). Scores of symptom severity were: 0, no lesions; 1, lesions found on leaf blades; 2, lesions found on petioles and leaf blades; 3, lesions found on young stems (including "sheperd's crooks"), as well as on petioles and leaves. The proportion of blighted leaves was estimated using the following classes: 0, no infection; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. Then weights of 5, 15, 23 and 33.3 were assigned to each of the last four categories, respectively (Newcombe and Oosten 1997). Multiplication of symptom scores and severity weights resulted in a scale ranging from zero (highly resistant) to 100 (highly susceptible).

In vitro screening for resistance to *S. musiva* and *S. populicola* was carried out using leaf and stem assays at the United States Forest Service (USFS) laboratories, North Central Research Station, in St. Paul, Minnesota. For the leaf assay, fully expanded leaves numbered 3, 4 and 5 (counting down from the shoot apex) were collected from three ramets of each transgenic and control line, all between 2-5 months in age. A total of 12 leaf discs (from each ramet) 18 mm in

diameter were taken using a cork borer; six discs were used for inoculation with each pathogen. The discs were placed in holes made in 2% water agar in petri dishes, with the abaxial surface facing up. A suspension prepared from 7- to 10-day old cultures was made using distilled water to give a concentration of 1×10^6 conidia/ml. Leaf discs were inoculated with either 100 μl of the conidial suspensions or deionized distilled water, and incubated at 20-25 °C under continuous light for 32 days. Inoculation experiments were repeated 2 to 3 times over a period of 4 months. Disease development was measured beginning on the 4th day following inoculation using a dot grid, where percentage of green leaf tissue was determined at the end of the 32nd day. A high percentage of green tissue indicates high resistance. Lines having >50% mean healthy leaf tissue (HLT) were considered to be resistant.

Stem assays for *Septoria* canker were carried out by removing three petioles from each of 2- to 3-month old ramets of transgenic and control trees, to expose inoculation sites. Agar sections (9 mm^2) bearing spores were taken from 7- to 10-day old cultures of *S. musiva* and *S. populicola*, and applied to wounded sites on each of two petioles for every tree. A section taken from pathogen-free agar was applied to the third petiole. Wounds were sealed with parafilm and canker formation was measured (in cm^2) 12 weeks after inoculation. Larger canker size indicates higher susceptibility. The experiment was conducted only once.

Northern analysis and hybridization probes

The shoot apex and the first expanding leaves were collected from 3- to 4-month old plants for total RNA extraction following the method of Hughes and Galau (1988). Northern blots were made according to standard protocols (Sambrook et al. 1989), and probes were radioactively labeled (^{32}P) using a nick translation labeling kit (Pharmacia Biotech). Radioactive membranes were analyzed using a phosphorimager (Molecular Dynamics) and RNA bands

quantified using ImageQuaNT (version 4.2). The poplar phenylalanine ammonia-lyase (*PAL*), *PAL7* cDNA, designated as *PAL1* was provided by Dr. Carl Douglas, University of British Columbia, Canada (pPAL7, Subramaniam et al. 1993). Poplar wound-inducible (*win*) chitinase genes, *win6* and *win8*, were provided by Dr. John Davis, University of Florida (pWin6 and pWin8FL, Parsons et al. 1989). Poplar nuclear 18S rDNA in pPD5 was used to estimate RNA loading (D'Ovidio et al. 1991). Except for the pEL301 (as described above) all of the genes were subcloned in pBluescript (Stratagene) and maintained in *E. coli* DH5α (Gibco BRL).

RNA samples (20 μg) were run in formaldehyde agarose gels and blotted (BioRad). Two RNA blots (A and B), were prepared for running samples from all of the transgenic lines and control plants. Blot A contained samples of lines 16, 17, 20, 26, and 28 from clone 189-434; lines 3, 4, 6, 8, 13, 41, 43, and 47 from clone 195-529; and lines 29, 31, 33, and 38 from clone 311-93. Blot B contained samples of lines 17, 19, 21, 22, 23, 24, 25, and 27 from clone 189-434; lines 1, 2, 5, 7, 10, 11, 12, 42, 44, and 45 from clone 195-529; and lines 32, 35, 36, and 37 from clone 311-93. RNA samples for control plants (C1 from clone 189-434; C2 from clone 195-529; and C3 from clone 311-93) and transgenic line 17 were included on both blots. RNA gel-blots were hybridized sequentially with *bO* (761 bp *Hind*III - *Sst*I fragment of pEL301), *PAL1* (994 bp *Sac*I - *Xho*I fragment of pPAL7 cDNA), *win6* (a 725 bp *Eco*RI fragment from pWin6), *win8* (a 770 bp *Eco*RI fragment from pWin8FL) and 18S rDNA (a 1.1 kb *Sac*1 - *Xba*1 fragment from pPD5). Washing and stripping of probes followed the manufacturer's protocol (BioRad). Hybridization was performed at 43 °C in 50% formamide/0.12 M Na_2HPO_4/0.25 M NaCl and 7% SDS at pH 7.2. Three sequential washes were done at 43 °C in 2X SSC/0.1% SDS, followed by washes at 55 °C in 0.5X SSC/0.1%SDS and at 55 °C in 0.1X SSC/0.1%SDS. Probes were stripped in 0.1X SSC/0.5%SDS at 95 °C. RNA bands were quantified as before. Expression from line 17 was used to standardize *bO* expression after correction of all samples based on rDNA hybridization (because of its low *bO* expression, its relative expression was set to

1.0). To standardize *PAL1* expression, the lowest *PAL1* expressers on each blot were set as the reference 1.0 unit (line 43 for blot A and line 32 for blot B).

Because of very low expression of the chitinase genes studied in both transgenic and non-transgenic poplars, their expression was induced through mechanical wounding. Transgenic lines 6, 41 and 12 from clone 195-529 were selected for study. They represented high, moderate, and low levels of HR-lesion intensity, respectively. Two ramets from each line and its non-transgenic control (C2) were grown in a greenhouse for 5-6 months. One ramet was wounded prior to RNA extraction, and the other ramet was left unwounded. Leaf numbers 5 to 8 (where 1=newly expanding leaf at shoot apex) were chosen for wounding. Using a small pair of pliers, half of each leaf (divided by the mid-vein) was wounded with a total of 15-20 "bites" at 0, 4, 17, 20 and 24 h. After 36 h following the first wounding treatment, the undamaged half (D) of each leaf was harvested. For controls, half of each leaf from the same specified leaves, were collected from undamaged trees (UD). In addition, RNA was extracted from shoot (shoot apex and the first expanding leaf) of the high *bO*-expressing line 6, and its control (C2). The RNA extraction and northern analysis procedures used are described above. The RNA blot was probed sequentially with *win6*, *win8*, *PAL1*, *bO* and 18S rDNA. Expression of all 4 genes from the UD control tree line (C2) were used to standardize gene expression after correction of all samples based on rDNA hybridization (relative *win6*, *win8*, *PAL1* and *bO* expressions were set to 1.0).

RESULTS

Lesion formation and growth

We produced 12, 16 and 7 independent transgenic lines from clones 189-434, 195-529 and 311-93, respectively (Appendix B). They were resistant to

kanamycin, verified as containing *bO* via PCR, and *bO* expression was confirmed via northern analysis (see below). Lesions started to develop in the greenhouse 4-8 weeks after transfer into soil and continued in the field (Figure 2.1A and B). Most of the lines exhibited few or no lesions. However, from our preliminary observation in the greenhouse, line 41 showed frequent visual lesions wherelse line 6 had consistently high lesion development. Therefore, we selected these two lines to represent moderate and high lesion expressers from clone 195-529. After being transferred to the field, the transgenic lines displayed phenotypes similar to what

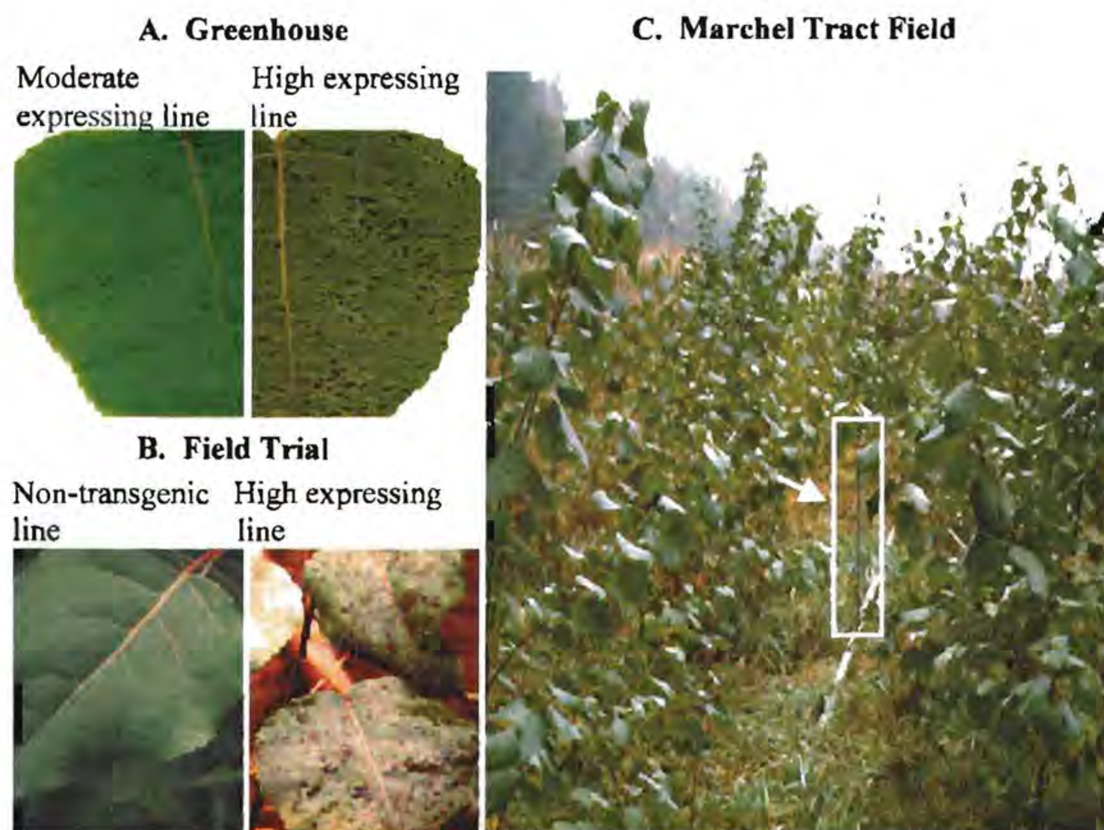

Figure 2.1 Expression of spontaneous lesions on leaves from trees grown in the greenhouse (A) and field (B). The left panel in A shows leaves from the moderate *bO*-expressing line 41. The left panel in B is its non-transgenic counterpart (clone 195-529). The right panels (in A and B) are the high *bO*-expressing line 6. (C) Trees after one growing season in the field at Marchel Tract, Corvallis (photo taken in August, 1998). The arrow and box show a sprinkler pipe with an approximate height of 1.2 m.

was observed in the greenhouse. To reduce the confounding effect of insect attack and other biological factors on lesion formation in the field, we assessed lesion frequency during the following growing season, in early spring 1998; as new leaves developed. Severe lesions continued to develop in line 6, moderate lesions in line 41, and there was no obvious differences in lesion development between any of the other transgenic lines, as compared to the control plants. Distribution of lesion density among all of the transgenic and control lines is depicted in Figure 2.2.

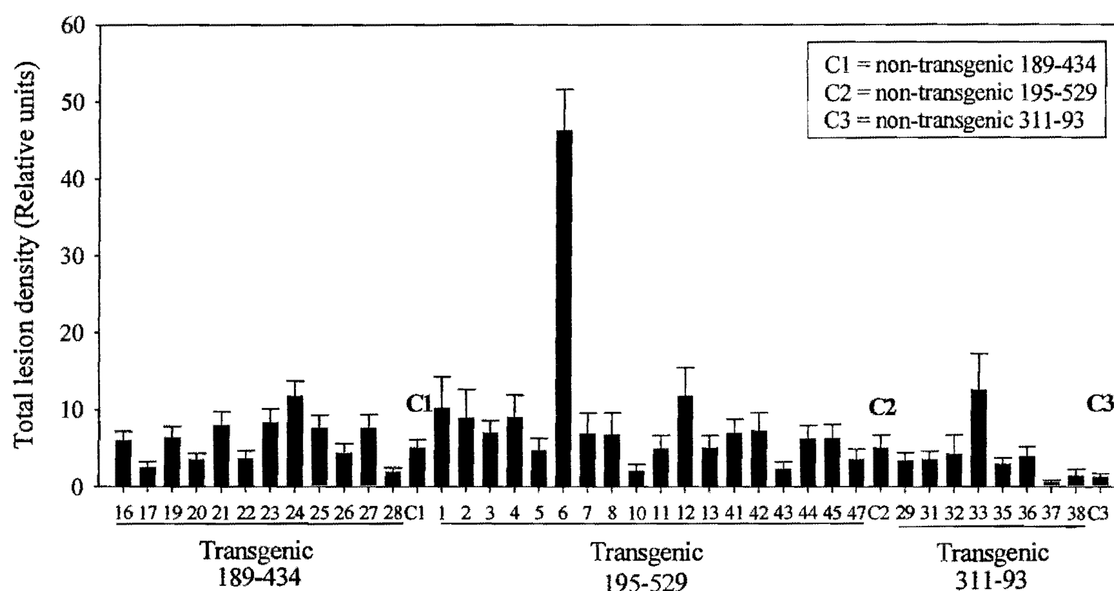

Figure 2.2 Mean lesion density for all transgenic and control lines as scored prior to inoculation study. Data are mean TLD from a total of 6 ramets for each line, grown at the two field sites. One SE of the mean is shown above bars.

Lesion formation was striking in all ramets of line 6, but was not consistent in the different ramets of line 41 (Figure 2.3).

Lesion density varied significantly with leaf age ($p < 0.00$; Appendix C). In clone 195-529, leaves 6 and 7 had higher lesion densities compared to leaves 2 and 3 or 4 and 5 (least-squares means (LSM) analysis; Appendix C). In the high *bO*-expressing line 6, lesions were visible as early as leaf number 2, and lesion density

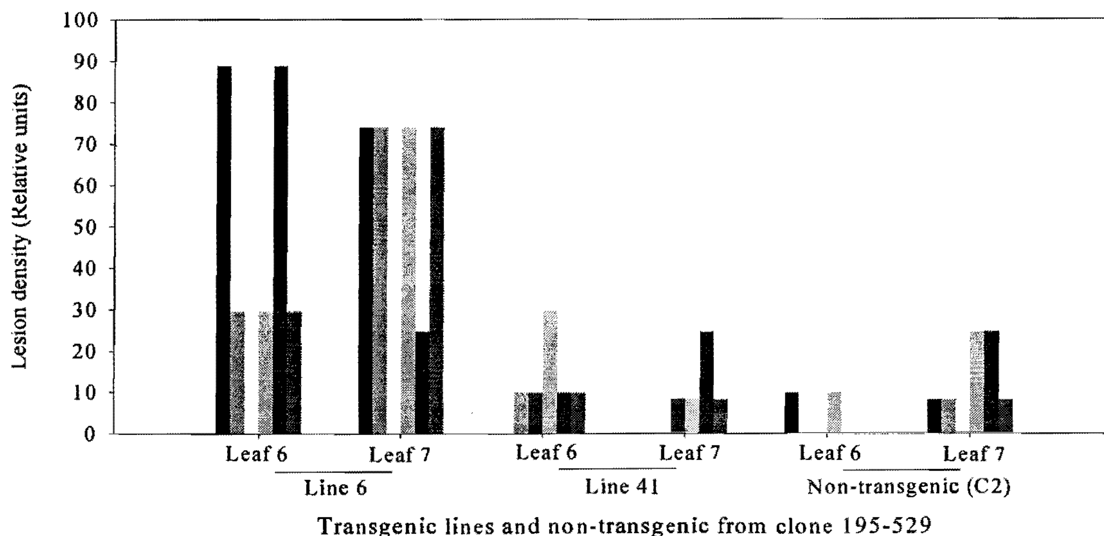

Figure 2.3 Lesion density determined on leaf numbers 6 and 7. Data are from high (line 6) and moderate (line 41) *bO*-expressing lines, including the non-transgenic control (C2) from clone 195-529. Each line and control consists of 6 trees; a column represents one tree. Missing columns in line 41 and non-transgenic indicate that lesion density values were zero; one missing column in line 6 was due to a dead tree.

gradually increased as leaves aged and then plateaued at about the stage where leaves began to senesce (Figure 2.4). Because line 6 was stunted in growth, only a few leaves were formed (less than 15 leaves per tree). Lesions in line 6 varied in shape and size, and were usually brown in color. They appeared at margins of the leaves or scattered across the surface. Older leaves exhibited chlorosis and eventually senesced. In contrast, the pattern of lesion formation seen in line 6 was not obvious in line 41, which represented moderate rate of lesion formation. The degree of necrotic spots was less severe, necrosis was mostly confined to the middle or lower leaves, little chlorosis was seen on older leaves, and the severity appeared to vary substantially between ramets. The other transgenic lines were all similar to the control plants; they showed few or no lesions (Figure 2.2). No lesions were detected on stems or petioles in any of the plants.

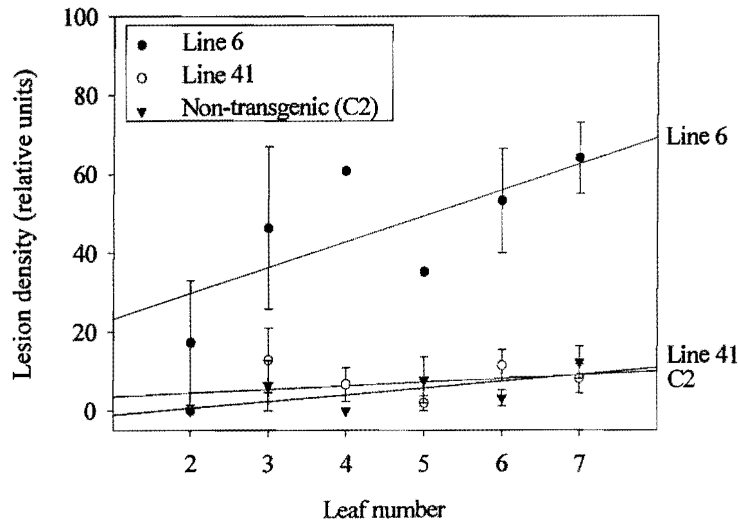

Figure 2.4 Lesion density determined from leaf numbers 2 to 7 (leaf 1 = first fully expanded leaf on shoot terminal). Data are from the high (line 6) and moderate (line 41) *bO*-expressing lines, including the non-transgenic control (C2) from clone 195-529. Bars represent two standard errors of the mean over 6 ramets. Points without bars are a result of a lack of variation in lesion score, thus no SE could be calculated.

ANOVA of mean TLD showed that clones did not differ significantly in lesion severity ($p < 0.11$; Appendix C); however, lines within a clone differed strongly ($p < 0.00$). The variation among lines within a clone was a consequence of the very high lesion severity in line 6; it had 9-fold higher lesion density than its corresponding control line. None of the other lines were significantly different from their control lines (Dunnett's test, α=0.05; Appendix C). Mean TLD for line 6 was 46 (± 5.9), and ranged between 0.3-12.5 for the other lines, including the control plants (Appendix C). For line 41, which represented the group, which had the next highest lesion severity after line 6, mean TLD was 7 (± 1.9).

Analysis of net growth after a year in the field showed that the trees planted at MT grew significantly faster than those at PA ($p < 0.00$; Appendix D); mean increment in volume index at MT was almost 180-fold greater than that at PA. There was no statistically significant difference in growth between the clones (p <

0.17). The interaction between site and clone was non-significant ($p < 0.32$). There was evidence that expression of the *bO*-transgene affected growth ($p < 0.02$). Using Dunnett's test, we found strong evidence that line 6 was retarded in growth, compared to its control (C2) from clone 195-529. Based on the logarithmic net growth, C2 grew 10.3 times faster than line 6 ($p < 0.00$, Dunnett's test; Appendix D). Visual inspection also showed line 6 was by far the smallest among all the transgenic lines; its absolute net growth was 1,500 cm^3 (± 1,071), whereas the growth of C2 was 117,400 cm^3 (± 70,300 cm^3), about 78-fold higher. No other transgenic lines were significantly different in growth rate compared to their control plants. Lines within clones grew slightly different at the two sites ($p < 0.05$, for line and site interaction).

Northern analysis

Total RNA was extracted from shoots of plants that had been growing for 3-4 months since transplanting from tissue culture into pots in the greenhouse. All 35 transgenic lines under study (Appendix B and E) were confirmed to be expressing *bO* mRNA. A band of the expected size (761 bp) was observed for transgenic lines but not for control plants (lanes labelled C1, C2 and C3) (Figure 2.5). Two lines selected as being kanamycin resistant and contained *bO* gene as verified through PCR were shown to lack the *bO* mRNA based on northern analysis (lanes labelled 3 and 4, Figure 2.5; line 35 is not shown). To quantify the amount of total RNA loaded for each lane, we probed with the gene encoding 18S rRNA (rDNA) isolated from poplar. Standardized against 18S rDNA, the levels of *bO* expression varied approximately 33-fold among transgenic lines (10-fold among 16 lines in clone 195-529; 13-fold among 7 lines in 311-93, and 33-fold among 12 lines in 189-434). Despite high levels of *bO* expression in many lines, lesion development

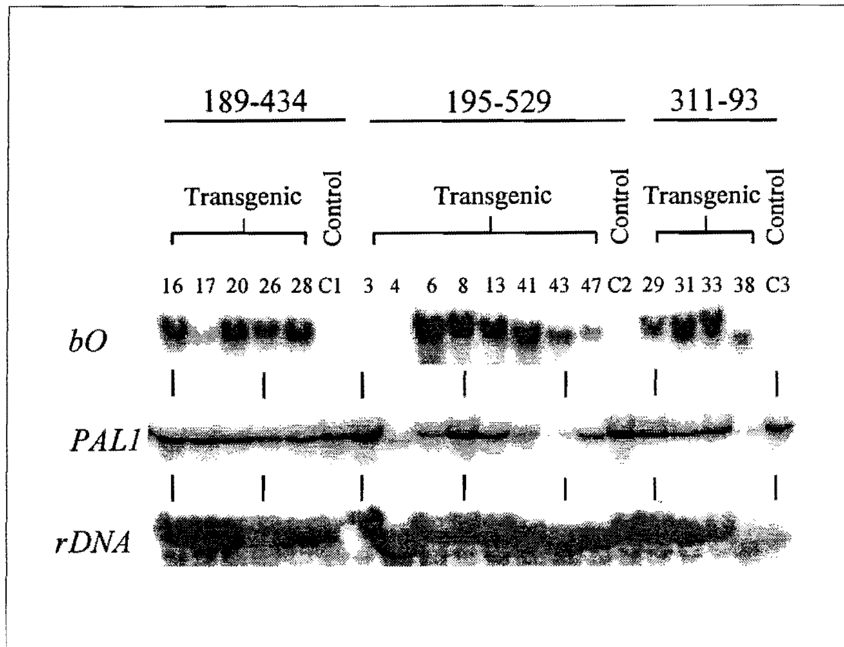

Figure 2.5 Northern blot analysis of poplar transgenic lines expressing bacterio-opsin (*bO*) and phenylalanine ammonia-lyase (*PAL1*) genes.

above that of controls was only observed in one line (6). Because the moderate (line 41) and high (line 6) lesion-forming lines were from clone 195-529, we regressed standardized *bO* expression of all the transgenic and control lines in this clone against lesion severity; there was no significant correlation between these two variables ($r=0.31$, $p < 0.18$; Appendix E).

To determine whether the transgene triggers expression of defense-related genes, we probed the RNA blots with *bO*, *PAL1* and two different wound-inducible chitinase genes (*win6* and *win8*). The *bO* gene did not induce increased *PAL1* expression among *bO* transgenics, as compared to controls. In fact, it appeared to do the opposite, though the difference was non-significant (t-test, $p < 0.08$; Appendix E). *PAL1* and *bO* levels were also not statistically correlated ($r = 0.00$, $p < 0.99$ for blot A; $r = -0.13$, $p < 0.53$ for blot B; Appendix E) (Figure 2.5). The mean level of *PAL1* expression for *bO* transgenics was 8.8 (± 1.3) and that of the controls was 14.6 (± 2.0). Because the level of expression of the chitinases studied

was nearly undetectable in the greenhouse-grown plants, we induced expression via mechanical damage. For this study, we used four lines from clone 195-529 with variable lesion formation. We selected transgenic lines 12, 41 and 6 to represent low, moderate and high levels of lesion formation (from our initial greenhouse observation), and the non-transformed (C2) as control. We studied the expression of *bO*, *PAL1*, *win6* and *win8* before and after induction. The four genes had different levels of induced expression ($p < 0.00$; Figure 2.6; Appendix F).

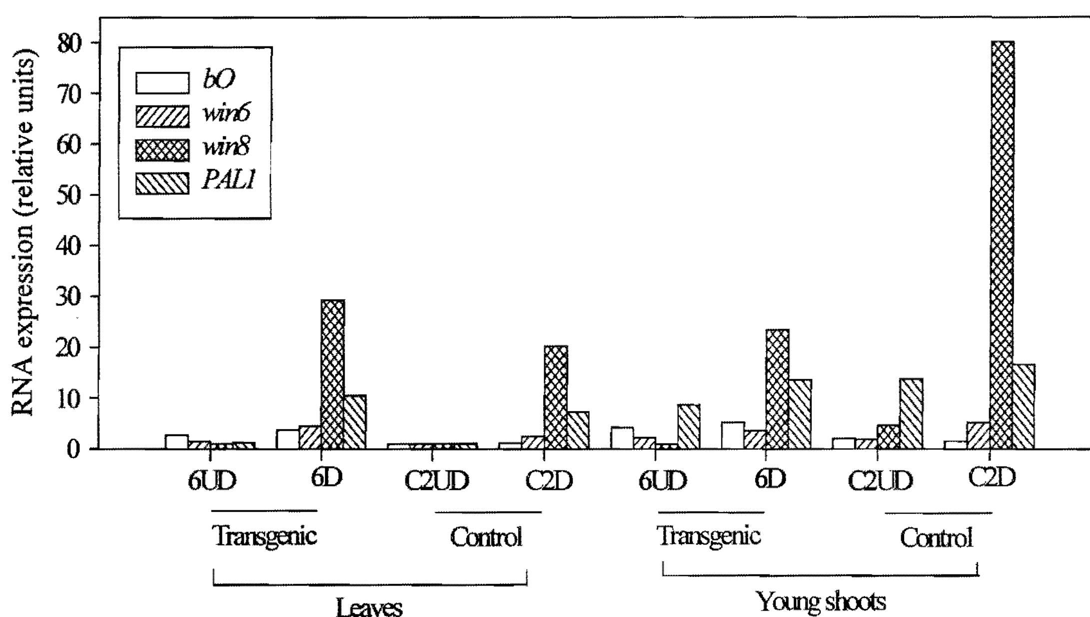

Figure 2.6 Expression of several defense-related genes after induction via mechanical wounding. RNA samples were collected from leaves and shoot apices of damaged (D) and undamaged (UD) trees. Data are from line 6 and its non-transgenic (C2) line from clone 195-529.

Wounding up-regulated expression greatly (mean of 1.2-fold in leaves for *bO*, 2.3-fold for *win6*, 19.5-fold for *win8*, and 4.4-fold for *PAL1*) (Appendix F). Apart from *bO* expression, these increases were highly statistically significant (Appendix F). However, the transgenics did not differ from controls in expression levels for *PAL1*

or any of the *win* genes, whether considered before or after induction. Even for the high *bO*-expressing line 6, there were no significant differences in *PAL1* or *win* gene expression before or after induction (Figure 2.6).

Effect of *bO* expression on resistance to fungal pathogens

Venturia *leaf and shoot blight*

We conducted artificial inoculation in the field and assessed disease severity (Appendix C). Disease symptoms were 1.4-fold greater on the trees in MT compared to those in PA ($p < 0.00$; Appendix C). The three clones also differed in their degree of susceptibility ($p < 0.05$). Clone 195-529 was the most highly susceptible to the race of blight studied of the three clones studied (Figure 2.7). In clone 195-529, *Venturia* symptoms appeared as early as 10 days post-inoculation and progressed very rapidly. At early stages of infection, dark necrosis appeared on young leaves, especially at the margins, resulting in curling. Necrosis could also be seen as black patches on older leaves. Eventually infected leaves became severely necrotic and died, with necrosis spreading into petioles and terminal shoots, leading to "sheperd's crook", a hallmark of *Venturia* infection in poplar. In contrast, in clones 189-434 and 311-93, the disease was confined mostly to the leaves.

Transgenic trees exhibited nearly the same blight severity as control plants in all clones (Dunnett's test, $\alpha=0.05$; Appendix C). Clone 195-529 was most susceptible, with its mean severity (33 ± 1.8) approximately 5- to 7-fold greater than the other two clones (clone 189-434 = 5 ± 0.2; clone 311-93 = 6 ± 0.4). The lines performed similarly at both sites ($p < 0.05$).

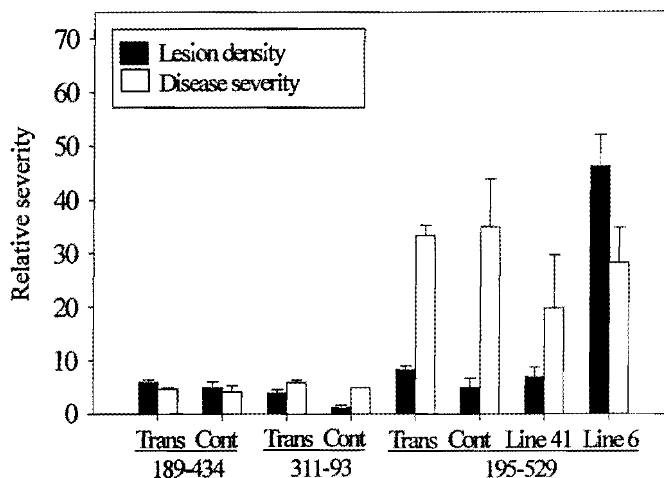

Figure 2.7 Lesion development prior to inoculation, and disease development following artificial inoculation with fungal spores causing *Venturia* shoot blight. Data are means over two field test sites; control (cont) bars are based on 6 trees and transgenic (trans) bars are based on 6 ramets per line and 7-18 lines per clone. One SE of the mean is shown above bars. There is no error bar for disease severity for the control of clone 311-93 because there is no variation; all 6 ramets had the same score.

Although the *bO* gene was moderately expressed in line 41 and highly expressed in line 6 of clone 195-529, and line 41 showed a strong propensity for lesion formation, they did not show significantly greater resistance to *Venturia* than the other transgenic lines or the control lines (Figure 2.7).

Melampsora *rust*

We selected a number of *bO* transgenic lines from the three clones and challenged them with *Melampsora* rust through artificial inoculation (Appendix G). Fungal spores appeared as early as 7 days after inoculation in some plants, in the form of orange pustules containing urediniospores on both sides of the leaves. The three clones varied in their degree of resistance towards rust infection ($p < 0.04$; Appendix G). Clones 195-529 and 311-93 were both highly resistant to rust with mean rust severity scores below 10 (the cut-off value for resistance is usually

considered to be <25; Schreiner 1959). No new orange pustules developed or spread on the leaf surface in these two clones 10 days after inoculation. However, clone 189-434 was very susceptible to rust; its mean rust-severity was 36 (± 7.2) for the transgenics, and 38 (± 15.3) for the control plants. Transgenic lines within a clone had no difference in resistance against rust ($p < 0.64$). The moderate (line 41) and high (line 6) *bO*-expressing lines appeared to be 2.5- and 4.5-fold more resistant to rust than the control line from clone 195-529. However, the modest absolute difference in rust severity between these two lines, as well as between all the transgenic lines and the control lines, were not statistically significant (Dunnett's test, $\alpha=0.05$; Appendix G). In clone 189-434, both the transgenic and control plants were highly susceptible to rust infection, all transgenics performing nearly the same as control plants in all clones (Dunnett's test, $\alpha=0.05$; Appendix G).

Septoria ***leaf spot and stem canker***

We tested resistance of the *bO*-poplars to *S. musiva* and *S. populicola* via *in vitro* inoculation (Appendix H). Results from the leaf assay showed that transgenic and control plants from all three clones were susceptible (≤ 50% HLT) to both *Septoria* spp., however clonal differences in susceptibility were apparent (Figure 2.8). All clones were more susceptible toward *S. musiva* than to *S. populicola.* Clone 195-529 was the least susceptible to *S. musiva* and clones 189-434 and 311-93 displayed about the same level of susceptibility. Clone 189-434 was the most susceptible clone to *S. populicola.* There was no difference between transgenic and control lines in their susceptibility to either fungus.

Stem assays resulted in similar findings. Clonal differences were significant, with clone 189-434 the most susceptible to *S. musiva,* and clone 311-93 the most susceptible to *S. populicola.* The two fungi caused similarly sized cankers

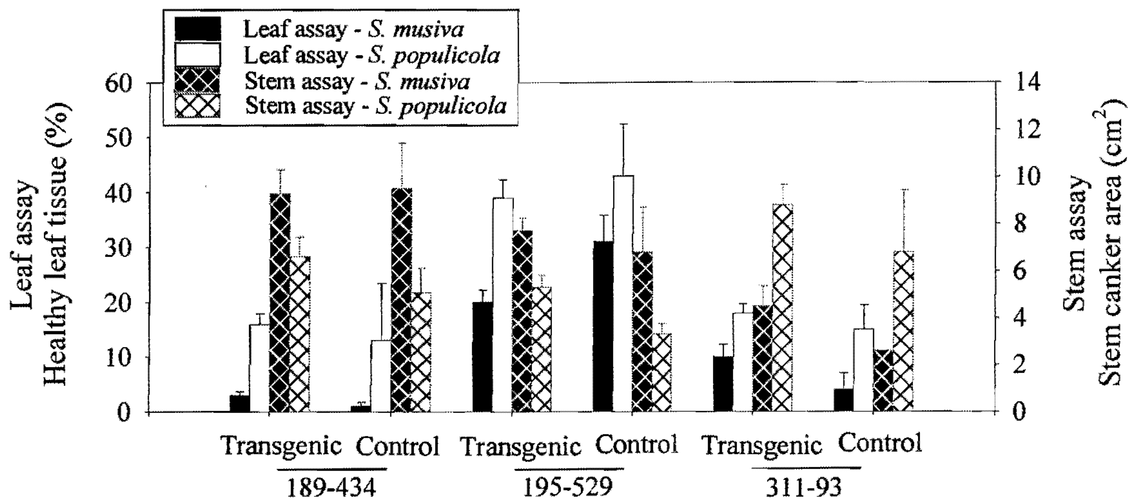

Figure 2.8 Resistance of transgenic and non-transgenic poplar to *Septoria* leaf spot and stem canker diseases after *in vitro* inoculation.

in clones 189-434 and 195-529. There were no differences in canker susceptibility between the transgenic and control plants in any of the three clones towards both fungi (Figure 2.8).

Discussion

Development of spontaneous lesions and effect on growth

We produced transgenic hybrid poplars containing the *bO* gene from *H. halobium*. Our observation showed that over-expression of the *bO* gene caused formation of spontaneous HR-like lesions in at least two lines. High expression of the *bO* gene, as in line 6, caused very severe lesions. This line consistently exhibited heavy lesions both in the greenhouse and in the field, and its growth was very slow compared to the other transgenic and control lines. Growth of the other transgenic lines, however, and lesion development, were not significantly different from the control plants despite constitutive, high levels of *bO* expression in most of them.

Lesion phenotypes of the *bO*-poplar leaves were similar to *bO*-expressing tobacco and potato; however, lesions were also observed on stems of the later two species (Mittler et al. 1995, Abad et al. 1997). Lesions also formed on flowers, but were not detected on tubers or roots in the *bO*-potato. Although growth of the *bO*-expressing tobacco plants was normal, some stunting was reported in the *bO*-expressing potato (Mittler et al. 1995, Abad et al. 1997). Furthermore, *bO*-tobacco plants transformed with a *bO* gene construct containing a stronger promoter, the figwort mosaic virus (FMV) 35S instead of the CaMV 35S promoter, were also stunted (Abad M.S., unpubls.). Together with our own findings, it appears that very high levels of *bO* expression can cause substantial growth reduction.

Induction of defense-related genes

The HR, which is commonly manifested by the formation of lesions, is associated with the induction of several general defense mechanisms, as seen from the study of incompatible host-pathogen relationships (Hammond-Kosack and Jones 1996). Because the *bO* gene induced HR-like lesions, it may also activate general defense genes. This was shown to be true when transgenic tobacco and potato plants expressing the *bO* gene exhibited elevated expression of defense genes, similar to that observed under microbial attack (Mittler et al. 1995, Abad et al. 1997). Two PR genes, chitinase (*PR-3*) and β-glucanase (*PR-2*), were induced in both species in response to *bO* over-expression. Although not studied in potato, the expression of *PR-1* (an anti-viral compound) and *PAL* (a precursor in the biosynthesis of phenolic compounds) were increased in the transgenic tobacco. The expression of PR genes that encode an osmotin-like protein and an anionic peroxidase were up-regulated in transgenic potatoes. Production of SA, a molecule that is associated with SAR, was also highly elevated in both species of *bO*-transgenic plants. The expression of several PR and *PAL* genes, with the exception of *PR-2* in transgenic tobacco, was higher in old leaves (with lesions) compared to

young leaves (no lesions) (Mittler et al. 1995). In addition, SA accumulated in both lower and upper leaves, suggesting that the induction of defense genes in the upper leaves was mediated through a SAR mechanism (Mittler et al. 1995). Contrary to findings of Mittler et al. (1995), Abad et al. (1997) reported that the SA content in young leaves of *bO*-potatoes was not affected consistently.

In contrast, we demonstrated that *PAL1* expression was not elevated in the *bO*-poplars, even in the high *bO*-expressing line, when compared to the control plants. In addition, the expression of two wound-inducible chitinases, *win6* and *win8*, was very low in *bO*-poplars, and did not differ from control plants. Although mechanical wounding successfully elevated their expression, transgenic and control plants showed similar amounts of induction.

Several gene products are induced by wounding and pathogens, including phytoalexins, chitinases, proteinase inhibitors, and PAL (Lawton and Lamb 1987, Bowles 1990). Two *PAL* genes (*PAL1* and *PAL2*) have been isolated from *P. trichocarpa* x *P. deltoides* (Subramaniam et al. 1993), and four other *PAL* genes (*palg1*, *palg2a*, *palg2b* and *palg4*) were identified in *P. kitakamiensis* (Osakabe et al. 1995). Both *PAL1* and *PAL2* are highly expressed in young leaves and stems (Subramaniam et al. 1993). Although *PAL* expression has been induced in fungus-infected parsley leaves (Reinold and Hahlbrock 1996), none of the *Populus PAL* genes have yet been studied for their role in disease resistance.

Three chitinase genes, *win6*, *win8* and *chiX*, have been isolated from poplar (*P. trichocarpa* x *P. deltoides*). They are responsive to wounding and were isolated from leaf tissues (Parson et al. 1989). Besides wounding, other stresses like viral, bacterial and fungal pathogens can also induce chitinase production, and many chitinase genes have been demonstrated to hydrolyze fungal cell walls *in vivo* (Collinge et al. 1993). Chitinases have been divided into five classes in tobacco, defined by their protein structures: class I, II, III, V and VII (Bol et al. 1996). Two of them, classes I and V, have antifungal activity *in vitro*.

Chitinases including classes IA, IB and II, are enzymes from plants that function in the defense against fungal and insect pathogens by destroying their chitin-containing cell wall (Flach et al. 1992; http://afmb.cnrs-mrs.fr/~pedro/CAZY /ghf.html). The win6 protein is predicted to have a structure similar to class I chitinases, which have the ability to hydrolyze chitin (Collinge et al. 1993, Clarke et al. 1994). When we searched the Pfam HMM database (http://pfam.wustl.edu/ hmmsearch.shtml) for similarities in amino acid sequences to win6 and win8, both proteins had strong similarities to class IA chitinases (expectant values (E) for win6 was $< 1.1e^{-153}$; and for win8 was $< 3e^{-164}$). Poplar win6 and win8 enzymatic activities were also grouped as EC 3.2.1.14, chitinases that can hydrolyze the β-1,4-N-acetyl-D-glucosamine in chitin polymer (PROSITE database; http://www. expasy.ch/cgi-bin/nice doc.pl?PDOC00620). Thus, these genes are likely to play a role in pest resistance in poplar; however, this has not been confirmed *in vivo.*

Resistance to pathogens

Transgenic tobacco and potato plants containing the *bO* gene exhibited a heightened resistance to a variety of pathogens. Mittler et al. (1995) challenged *bO*-expressing tobacco plants with *P. syringae* pv *tabaci.* Both the disease symptoms and replication of the bacterial pathogen were blocked. In addition, replication of TMV was successfully inhibited, and necrotic symptoms of TNV were blocked, as was the appearance of additional lesions. Because the viruses were inoculated on the top leaves and spontaneous lesions were confined to the lower leaves, the induction of resistance mechanisms was suggested to be through SAR. These findings were similar to the response of SAR-induced tobacco plants following pathogen infection (Ward et al. 1991). It was therefore suggested that the bO protein might act as an elicitor, with plants misinterpreting the bO protein as a pathogen, resulting in constitutive expression of several defense mechanisms (Mittler et al. 1995).

The effects of the *bO* gene on disease resistance can vary widely. Abad et al. (1997) challenged *bO*-expressing potato plants with several pathogens. The potatoes displayed enhanced resistance to the A1 (US1 isolate) mating type of *P. infestans* that causes late blight disease, and constituted the first report of *bO*-induced resistance to a fungal disease. However, it failed to confer resistance to the other mating type of late blight, A2 (US8 isolate). When challenged with the A1 mating type, new lesions on the upper leaves appeared during disease progression, although the older leaves had already formed *bO*-induced lesions. But when challenged with the A2 mating type, no additional lesions were formed; instead the potato leaves were rapidly consumed and killed. Both the *bO*-potato and control plants were equally susceptible to A2 mating type. The same phenomenon was observed when we challenged *bO*-poplars from a susceptible clone 195-529 with fungal spores causing *Venturia* leaf and shoot blight. The leaves were attacked very rapidly prior to the plants forming new HR-lesions. Abad et al. (1997) also studied the performance of *bO*-potato against the PVX and *E. carotovaro*, the bacterium causing soft rot in potato tubers. The transgenic potato plants were not resistant to either of the two pathogens. For *E. carotovora* infection, they suggested that protection failed because the systemic signaling mediated by *bO*-induced SAR could not penetrate into the tubers. For PVX, the *bO*-potato plants actually appeared more susceptible to PVX than the control plants. This suggests that different viruses have different type of pathogenicity, which cannot always be overcome, and may even be exacerbated, by *bO*-induced resistance (Abad et al. 1997).

In our study, we tested resistance of *bO*-poplars to four different fungal pathogens. To our disappointment, there was no observable difference detected in resistance between the *bO* and control plants. All were equally susceptible, but some variation in clonal susceptibility to the pathogens was observed. When challenged with *Melampsora* rust, transgenic lines from clone 189-434, which was very susceptible to rust, did not produce any additional lesions. When *bO*-poplars of the *Venturia*-susceptible clone 195-529 were challenged with *Venturia* blight, it

caused necrosis on leaves, petioles, and stems, similar to that observed in *bO*-potato infected with *P. infestans* mating type A2 (Abad et al. 1997). Additional negative results were obtained when *bO*-poplars were assayed for resistance against two *Septoria* spp., which cause leaf spot and stem canker.

Although over-expression of the *bO* gene was evident from RNA analysis and formation of the spontaneous HR-lesions, induction of biochemical markers involved in induced resistance was not detected. This indicates that the *bO* gene was associated with the lesion phenotype but appeared to not activate defense-related genes, in contrast to findings in tobacco and potato. Because the bO protein is known to act as a proton-pump, it can cause biochemical imbalance in cells. The perturbed homeostasis may be misinterpreted by plant cells as pathogen infection and thus trigger the HR and other general defense mechanisms (Mittler et al. 1995). In poplar, induction of defense genes mediated by bO protein appeared to be unsuccessful. This may be because we did not monitor the affected PR genes. However, it is also possible that the reaction to *bO* maybe different in poplar trees than in herbaceous species. Perhaps the longevity of trees, which cause them to serve as hosts to many insects and microbial species, may cause them to "demand" a higher induction threshold before they devote major cellular resources to pathogen defense. Studies of additional plant species, defense genes, and inductive treatments will be necessary to test this hypothesis.

CHAPTER 3

EVALUATION OF A COPPER-INDUCIBLE GENE EXPRESSION SYSTEM IN TRANSGENIC POPLAR

ABSTRACT

We studied a copper-inducible gene expression system that was first described in transgenic tobacco for its function in hybrid poplar (*Populus trichocarpa* x *P. deltoides*). The system consists of a constitutively expressed, copper-activated transcription factor (*ACE1*), together with a hybrid promoter containing an *ACE1* binding site (MRE) and the –90 fragment of the cauliflower mosaic virus 35S promoter, both driving the *GUS* reporter gene. A control construct lacked the *ACE1* gene. Surprisingly, *GUS* expression occurred at high levels in the absence of the *ACE1* gene. When the *ACE1* gene was present, *GUS* expression occurred in response to very low levels of copper ion, and showed an idiosyncratic dependence on copper concentration. The system does not provide useful copper-inducibility in poplar, probably because poplar contains endogenous molecules that activate expression in the absence of exogenously applied copper.

INTRODUCTION

Several chemical-inducible promoters have been identified from plants, lower eukaryotes and prokaryotes (reviewed in Gatz and Lenk 1998, Jepson et al. 1998). Chemically regulatable promoters cloned from plants include the *PR-1a* from tobacco, and *In2-2* and *GST-27* from maize. The *PR-1a* promoter responds to benzothiadiazole and isonicotinic acid, while both the promoters from maize respond to the safener benzenesulfonamide. Promoters isolated from prokaryotic or lower eukaryotic organisms require the expression of two genes: one encoding a

regulatory protein and a promoter element to which it binds. Examples of two-component gene expression systems tested in plants include those regulated by tetracycline, dexamethasone, copper ion, lactose, and ethanol (reviewed in Gatz and Lenk 1998, Jepson et al. 1998).

Copper may be particularly a useful chemical for inducing gene expression in plants. It is non-toxic at low concentrations, is readily absorbed by plants, is already applied in the field as a fungicide, and it does not have the environmental and health concerns that would accompany use of an antibiotic or steroid, especially tetracycline and dexamethasone. However, as an essential plant chemical it may also be affected by endogenous copper and changes in plant physiology that affect copper metabolism (Mett et al. 1993). Thus, its effectiveness for regulating gene expression needs to be carefully evaluated under diverse conditions and in diverse plant species.

Mett et al. (1993) described a copper-dependent regulatory system adapted from the yeast copper-metallothionein regulatory system for controlling gene expression in plants. In this system, the copper-dependent *ACE1* transcription factor is under control of the cauliflower mosaic virus (CaMV) 35S promoter, and is thus constitutively expressed. The *GUS* reporter gene is under control of a 90 bp domain A of the CaMV 35S promoter, fused to the *ACE1* transcription factor-binding site, MRE. Copper binds to *ACE1*, causing a conformational change in the protein, allowing for copper-dependent transcriptional activation of the hybrid promoter. A tissue-specific version of the system has also been developed in *Lotus corniculatus* (Mett et al. 1996). When a tissue-specific promoter controlled expression of the *ACE1* gene, *GUS* reporter expression was directed to nodules or roots of *L. corniculatus* (McKenzie et al. 1998). McKenzie et al. (1998) also replaced the native promoter of the isopentenyl transferase (*IPT*) gene with the copper-controllable promoter and introduced the modified cassette into tobacco. The IPT protein plays a role in the biosynthesis of cytokinin; over-production of cytokinin leads to abnormal morphological changes. They demonstrated that

treating transgenic plants with Cu2+ solution could regulate cytokinin production in tobacco.

To evaluate the potential of the copper-controllable gene expression system to direct transgene expression in poplars, we tested the system of Mett et al. (1993) in hybrid cottonwood clone 189-434 (*P. trichocarpa* x *P. deltoides*). Contrary to expression in other systems, we report that activation of expression system was observed in the absence of added copper and when the ACE1 activating protein was absent.

MATERIALS AND METHODS

Plasmid constructs

The plasmid constructs of Mett et al. (1993) were provided by Dr. P. Reynolds (The Horticulture and Food Research Institute of New Zealand Ltd., Palmerston North, N.Z.). In the following text, the constructs are designated as CT: the control construct, pMB705, which contains the hybrid promoter, MRE::CaMV 35S -90, directing expression of the *GUS* reporter gene, but lacking the *ACE1* gene; and FT: the full construct, pMB711, which contains the hybrid promoter directing *GUS* expression and the constitutively expressed *ACE1* gene for copper-dependent transcriptional activation. Both constructs contain the *nptII* (kanamycin resistance) selectable marker.

Poplar transformation and PCR analysis

Constructs were inserted into hybrid cottonwood clone 189-434 (*P. trichocarpa* x *P. deltoides*) via *Agrobacterium*-mediated transformation (Han et al. 1999). Transgenic plants were selected based on rooting ability in the presence of

25 mg/L kanamycin. All transgenic lines and non-transformed poplars were propagated in shoot propagation medium (SPM; ½-strength Murashige and Skoog media, MS) supplemented with growth regulators (Han et al. 1999). Genomic DNA was isolated from 2- to 3-month old plants growing in tissue culture, utilizing a modified version of the mini-scale DNA preparation protocol (http://www.fsl.orst.edu/tgerc/dnaext.htm). Transformant lines regenerated from different calli were verified through PCR amplification of *nptII* and *ACE1* genes from plants transformed with constructs CT and FT, respectively (Appendix A). The primer sequences for amplifying *nptII* gene were: forward primer 5' TTCGTCCAGATC ATCCTG 3', and reverse primer 5' TTCTTTTGTCAAGACCG 3', which amplified a band of 343 bp. The primer sequences for amplifying the *ACE1* were: forward primer 5' CACACTGATGGTCCGCTA 3' and reverse primer 5'CAATA TCGTTTAGTGCTGTGTTC 3', which amplified a band of 532 bp.

Copper induction treatments

Leaf sections (approximately 1.0 x 0.6 cm) were excised from plants propagated in SPM medium for 1-2 months. Wherever possible, sections were made from leaves of similar size. Five leaf sections were gathered from one ramet grown in a Magenta box and placed in microfuge tubes. Each tube was filled with one of five different $CuSO_4$ concentrations (0, 5, 25, 50 and 100 μM) and allowed to stand overnight, then GUS activity was assayed histochemically.

For *in vivo* (whole plant) copper-induction analysis, plants were propagated in liquid SPM for 6 weeks, then transferred to a copper-depleted SPM medium (lacks ½-MS, which normally contains 0.032 μM $CuSO_4$ (Gibco, BRL) for three days. Plants were then transferred to SPM supplemented with 50 μM $CuSO_4$. After 5 days of growth, leaves were subjected to histochemical GUS assay. Control plants were not exposed to copper-containing media. By comparison, 0.15 μM $CuSO_4$ was present in the copper-depleted medium of Mett et al. (1993).

Two high expressing FT lines (line 41 and 47) were selected for fluorometric GUS analysis. The transgenic lines were regenerated in SPM, then transferred to copper-depleted medium. Each line was grown in three Magenta boxes with five ramets per box. A total of 30 leaf sections were excised, 10 sections from each box. Sections were randomly placed into microfuge tubes in triplicate, and incubated in a range of $CuSO_4$ concentrations (0, 5, 25, 40, 50, 60, 70, 80, 90 and 100 μM). Samples were incubated overnight, then the leaf sections were rinsed with distilled water, blotted on tissue paper, frozen in liquid nitrogen before protein was extracted immediately (see below). Protein samples were stored at –80 °C for 2-4 weeks until GUS analysis was undertaken. Leaf sections were also prepared as described above for histochemical GUS analysis.

Measurement of GUS activity

Histochemical GUS assay

Histochemical GUS assays were performed using 2 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) by the method of Jefferson (1987). *GUS* expression was observed after explants were incubated for 24 hours at 37 °C, followed by clearing with 70% ethanol. The level of expression was scored visually based on a scale from 0 to 5, where 0 = no blue color, and 1-5 indicated progressively greater levels of staining.

Fluorometric GUS assay

Protein extracts were prepared by crushing frozen leaf sections in a microfuge tube with a micro-pestle in an extraction buffer containing 50 mM NaH_2PO_4 pH 7.0/ 10 mM EDTA/0.1% SDS/0.1% Triton X-100/10 mM β-

mercaptoethanol. The supernatant was collected after centrifugation at 14,000 rpm for 5 min at 4 °C. A BioRad Protein Assay Kit was used to determine protein concentration. Fluorometric GUS assays were performed according to Jefferson (1987). GUS activity was calculated as the amount of 4-methyl umbelliferone (MU) produced per mg protein over a 30-min incubation period at 37 °C.

RESULTS

Seven CT transgenic poplar lines and ten FT transgenic poplar lines were produced. All were resistant to kanamycin, and were verified through PCR to contain the constructs pMB705 and pMB711, respectively, whereas non-transgenic control plants lacked the diagnostic PCR products. In preliminary analysis, histochemical *GUS* expression was observed in leaves of all CT transgenics over the complete $CuSO_4$ concentration range (0, 5, 25, 50 and 100 μM). CT lines exhibited high levels of GUS activity regardless of the $CuSO_4$ concentration, including the 0 μM copper control (4.3 ± 0.4 to 5 ± 0) (Table J.1: Appendix J). GUS activity was not observed in the non-transgenic plants. In contrast, a trend in *GUS* expression was observed in eight out of the 10 FT lines (Table J.1: Appendix J). Substantial GUS staining was observed when leaves were incubated without copper. Surprisingly, addition of 5 μM $CuSO_4$ decreased overall blue color. The highest level of GUS activity in the presence of copper was observed at 50 μM (2.1 ± 0.5); however, this level was similar to activity seen at 0 μM $CuSO_4$ (2.3 ± 0.6). Exposure of FT tissue to 100 μM $CuSO_4$ inhibited GUS activity (0.5 ± 0.2). Two of the high expressing FT lines (41 and 47) were also induced with 60, 70, 80 and 90 μM $CuSO_4$. Similar to the 100 μM results, expression decreased at $CuSO_4$ concentrations over 50 μM (Table J.2, Appendix J). Based on these results, 50 μM $CuSO_4$ was considered to be near the optimal concentration for copper induction for further studies.

We investigated response of transgenic lines and non-transgenic plants to copper ion uptake in whole plant copper-induction assays. The plants were regenerated in liquid medium, transferred to Cu-depleted medium, followed by medium supplemented with 50 μM $CuSO_4$. Histochemical staining revealed high levels of GUS activity in all CT lines, irrespective of growth conditions (4.6 ± 0.2) (Table J.3: Appendix J). Background GUS activity was not observed in the non-transgenic plants. FT lines displayed a similar level of GUS activity when induced with 50 μM copper (3.0 ± 0.6) and in the absence of copper (3.1 ± 0.5) (Table J.3: Appendix J). Lines 41, 47 and 50 displayed the highest expression levels. We found no significant differences of GUS activity between the CT and FT groups, regardless of their growing conditions ($p<0.05$; Appendix J).

Two highly expressing transgenic FT lines (41 and 47) were used for more detailed histochemical copper-induction, and were further characterized by quantitative fluorometric GUS analysis (Figure 3.1 and Table J.4: Appendix J). These two lines responded similarly to the different copper concentrations ($p < 0.12$; Appendix J), but copper concentration strongly affected GUS activity ($p <$

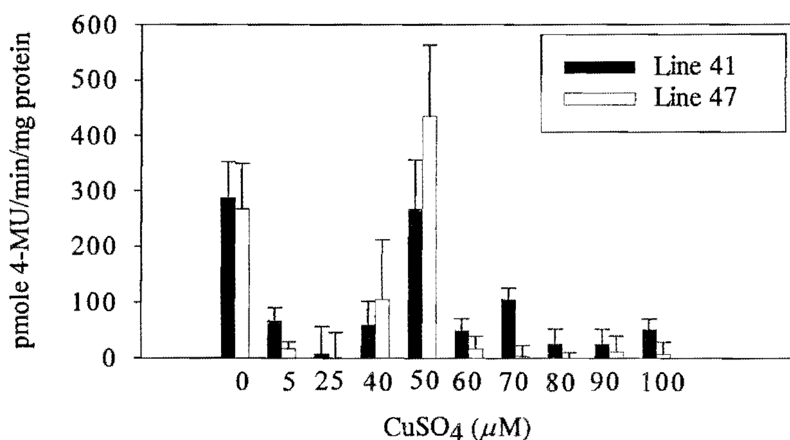

Figure 3.1 Effect of copper ion concentration on *GUS* gene expression in two transgenic poplar lines *(P. trichocarpa* x *P. deltoides* clone 189-434) containing the full copper induction gene-construct. One standard error of the mean from three replicates is shown above each bar.

0.00). Similar to the histochemical GUS results, the highest mean expression levels from these two lines were seen at 0 (277 ± 47.9) and 50 μM $CuSO_4$ (351 ± 79.1); low levels of copper supplement inhibited *GUS* expression through 25 μM (0 ± 37.7), then reversed and promoted *GUS* expression from 40 to 50 μM. At 60 μM and above, *GUS* expression was inhibited strongly; its expression was 12-fold higher at 50 μM than at 100 μM. The expression at the zero level of added copper was almost 10-fold higher than at 100 μM. *GUS* expression at 0 and 50 μM $CuSO_4$ was not significantly different ($p < 0.99$, Dunnett's test; Appendix J).

DISCUSSION

Regulation of the copper-inducible promoter in poplar differed from that reported in tobacco (Mett et al. 1993). Strong constitutive *GUS* expression was observed in seven independent poplar CT lines in both the presence and absence of copper. By contrast, in tobacco CT lines GUS activity was not observed in the absence of copper, and addition of copper did not affect *GUS* expression (Mett et al. 1993). The CT construct lacks the *ACE1* gene, which was expected to be necessary for induction of the copper-regulated promoter. In tobacco, the lack of induction in CT plants (Mett et al. 1993) verified that the presence of *ACE1* gene-encoded protein is essential for the system to function in tobacco. It appears that the ACE1 protein is not required for transcription from the MRE-minimal 35S promoter in poplar.

Constitutive *GUS* expression of the CT lines could result from an endogenous copper-independent transcription factor in poplar that binds to and activates the MRE in a manner analogous to that of the activated ACE1 protein. The hybrid promoter contains an Activating Sequence Factor 1 (*ASF 1*) binding site in the CaMV 35S -90 promoter fragment. In tobacco, constitutive *GUS* expression was detected in transgenic roots, but not in leaves (Mett et al. 1996). In tobacco,

the CaMV 35S -90 fragment targets gene expression predominantly to root meristematic tissue, and at low levels in leaf vascular tissue (Benfey et al. 1989). In poplar, however, the presence of this region may have caused more generalized expression in leaf tissue. Alternatively, the endogenous poplar factor may bind to another segment of the hybrid promoter to constitutively activate expression. In either case, binding to the metallothionein promoter would trigger constitutive reporter gene expression in the CT lines. Plants such as pea (Evans et al. 1990), soybean (Kawashima et al. 1991) and maize (de Framond 1991) contain native metallothionein-like proteins. In *Arabidopsis*, a single transformant containing the CT construct was observed to constitutively activate reporter gene expression in a fashion similar to the results reported here (V. Mett, pers. comm.). This single transformant may have been the result of an integration event near a constitutively active segment of the genome. However, only a single transformant behaved this way, whereas in poplars all of our CT transgenic lines showed constitutive *GUS* expression.

In the FT lines, *GUS* expression varied with copper concentration, but in a complex, idiosyncratic manner. Maximal induction was seen at both zero and 50 μM, and strong inhibition seen at 25 to 40 μM and 60 to 100 μM. The latter result may have been due to copper toxicity. Prolonged growth of tobacco plants at 50 μM $CuSO_4$ led to copper toxicity (Mett et al. 1993). However, the complex pattern of induction seen between 0 μM and 50 μM is likely to have a more subtle cause.

Competition between an endogenous poplar transcription factor and the ACE1 protein may explain the complex induction pattern in FT lines seen at low copper concentrations. The endogenous poplar factor may bind at or near the MRE, causing competition between it and the copper-activated ACE1. The endogenous factor's binding to the hybrid promoter appears to be independent of copper, as evidenced by the lack of dependence of *GUS* expression on copper in the CT transgenic poplars. At low concentrations of copper, active Cu::ACE1 protein complexes would start to form and compete with the endogenous factor for binding to the hybrid promoter. In FT lines where ACE1 protein is present, a reduction in

overall activity was observed following addition of 5-40 μM copper, suggesting that active Cu::ACE1 protein complexes are binding to the promoter and interfering with the endogenous factor. At 50 μM, however, there appears to be sufficient Cu::ACE1 to out-compete the endogenous factor and fully stimulate transcription. At higher copper concentrations (above 50 μM), reporter gene expression declines, perhaps as a result of copper toxicity.

Our results demonstrate that the copper regulated expression system of Mett et al. (1993) operates differently in hybrid poplar than in tobacco. In poplar, high expression occurs in the absence of the ACE1 protein, and in the absence of added copper. Induction kinetics between 5 and 40 μM were complex. The system tested therefore would not be useful as a simple means for control of gene expression in poplar. Mett et al. (1996), however, reported a new version of the copper-regulated promoter. The CaMV 35S -90 promoter fragment was truncated to -46, eliminating the *ASF-1* binding sites and maintaining just the TATA-box-containing fragment of the 35S promoter. Additionally, four repeats of the MRE, instead of one, were fused to the CaMV 35S -46 promoter fragment. With the new construct, constitutive background *GUS* expression in roots was reduced (Mett et al. 1996). This system should also reduce background expression in the absence of *ACE1* in poplar, but may still give a complex induction pattern if *ACE1* indeed interacts with an endogenous transcription factor.

CHAPTER 4

CONCLUSIONS

MAJOR RESULTS

1. The *bO* gene has been integrated successfully into poplar genome using *Agrobacterium* transformation. The transgene causes formation of spontaneous necrotic lesions on transgenic poplar leaves as observed in the moderate and high *bO*-expressing lines, which mimics HR-lesions in an incompatible host-pathogen interaction.
2. No obvious deleterious effects on growth were observed in the *bO*-expressing poplars, except in one high expressing line. The transgene appears to seriously impair growth only under high expression. Because only a single year of growth was studied, however, it is likely that longer-term studies would show growth impairment at lower levels of transgene expression as well. Therefore, a promoter system that can up-regulate *bO*-gene expression specifically during conditions conducive to disease development would likely be needed, in the event that *bO*-induced resistance had proven to be effective.
3. Levels of *PAL1* and wound-inducible chitinase genes, *win6* and *win8* were not elevated as expected, although *bO* over-expression was detected. Although these genes were isolated from poplar and are expected to play a role in pest resistance responses, based on studies in other species, there is no evidence for their direct involvement in disease resistance in poplar. We speculate that a higher threshold for triggering HR-related defense response genes may be required in tree species like poplar than in herbaceous plants, because trees are exposed to many different kinds of pests and pathogens during their life cycle.

4. The *bO* gene did not confer resistance to the fungal pathogens causing leaf and shoot blight (*Venturia*), rust (*Melampsora*), and leaf spot and stem canker (*Septoria*) diseases of poplar, possibly because the transgene was not able to up-regulate expression of defense genes.
5. The copper-inducible gene expression system failed to control gene expression in poplar as previously demonstrated in tobacco. Constitutive expression of the reporter *GUS* gene was observed in the transgenic poplar containing the control construct (*GUS*-MRE), regardless of copper ion presence or absence. This suggests that there is a native transcription factor in poplar, independent of copper influence, which interacted with the promoter studied and allowed *GUS* expression.

MAJOR CONCLUSIONS

1. Genetic engineering of disease resistance using the *bO* gene to elevate hypersensitive lesion development does not appear to be effective in poplar.
2. Transgenic methods have great potential for improving disease resistance, but require careful consideration to the types of genes utilized, and the nature of the host-pathogen interaction. The highly specific interactions between pathogens and plants are likely to require much more sophisticated transgenic approaches.
3. The copper-inducible gene expression system failed to control gene expression in poplar as previously demonstrated in tobacco. Gene induction systems often vary widely in effectiveness among species due to differences in physiology, requiring their careful testing, and possibly adoption, in different hosts.

FUTURE RESEARCH SUGGESTIONS

Gene transfer methods provide new options for breeding resistant plants. We introduced an HR-induction gene derived from a bacterium into poplar, but

failed to show any useful protection against fungal diseases. Many studies employing genetic engineering strategies have been reported. However, except for the resistance mediated by viral coat protein, induced resistance has largely been highly specific or did not provide field-level resistance, thus was not suited for commercialization. New approaches that emulate natural resistance mechanisms maybe more fruitful.

One option would be to isolate and introduce native resistance (R) genes isolated from poplar into susceptible commercial clones. Several diverse R genes could be employed, possibly from diverse poplar species. These should enhance durability of resistance. Many molecular tools have been developed to isolate R genes, and a number of R genes have been characterized from different plant species (Hammond-Kosack and Jones 1997, Parker and Coleman 1997). Future work should be aimed at isolating the thousands of R genes in poplar with conserved R gene sequence motifs. At present, a PCR approach based on amino-acid sequence similarity among different cloned R genes has been widely adopted for this purpose (Kanazin et al. 1996, Yu et al. 1996, and Leister et al. 1996). Many of the R gene homologs have been co-mapped to known resistance loci, leading to possible identification of the actual R genes.

Both R genes and other DNA markers can be employed to aid breeders via marker-aided selection (MAS). A dense genetic map has been established in poplar, containing several hundred genetic markers and QTLs, including QTLs governing resistance to poplar diseases (Bradshaw et al. 1994, Bradshaw and Stettler 1995, Newcombe et al. 1996, and Cervera et al. 1996). MAS is a selection procedure where the future phenotype (resistant or susceptible to a disease) of a young tree can be determined based on the genetic marker data, thus saving time as susceptible trees can be rejected immediately. However, linkage between genetic markers and disease resistance must first be established by finding genetic markers that display the same pattern of inheritance as disease resistance. Once linked markers have been identified, they can be used to rapidly screen large numbers of plants at low cost. The successful application of MAS strategies in genetically

diverse germplasm depends on the availability of markers that are very tightly linked to disease resistance genes, and preferably are within the resistance genes themselves. This requires either using catalogs of known R genes, or map-based cloning after QTL analysis using random markers. In either case, a physical map of the genome is important. A poplar bacterial artificial chromosome (BAC) library is now available, which contains 50,000 clones with an average insert size of 110kb. By comparing restriction-fragment patterns of the BAC clones, those that share the same pattern can be assembled into contigs, which as a unit represent a physical genome map. This resource will greatly speed the identification and isolation of R genes, whether for marker-aided breeding or genetic engineering.

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APPENDICES

APPENDIX A: PCR CONFIRMATION OF TRANSGENICS

Plants were maintained in tissue culture media (in Magenta boxes) until 2-3 months of age. Total genomic DNA was isolated from individual transformants using a small-scale DNA preparation protocol (http://www.fsl.orst.edu/tgerc/dnaext.htm, Bradshaw and Stettler 1993). PCR primers were synthesized by The Midland Certified Reagent Co., Texas.

A) Transgenic poplar expressing *bO* gene

Specific PCR primers were designed based on the synthetic *bO* sequence (Genebank Accession Number = J02755). The forward and reverse primers used are as follows:

Forward : 5' ATGCAAGCTCAAATTACTGGAC 3' (22-mer)

Reverse : 5' AAGATAGCTCGAGAACGCAG 3' (20-mer)

Expected amplification product = 692 bp

PCR conditions: Denaturation temperature = 94 °C for 1 min 30 sec

Annealing temperature = 53 °C for 1 min 30 sec

Extension temperature = 72 °C for 2 min

Cycles = 40

B) Transgenic poplar expressing the copper-controllable gene expression full-construct, pMB711

Specific PCR primers were designed based on the *ACE-1* sequence (Genebank Accession Number = M22580). The forward and reverse primers used are as follows:

Forward : 5' CACACTGATGGTCCGCTA 3' (19-mer)

Reverse : 5' CAATATCGTTTAGTGCTGTGTTC 3' (23-mer)

Expected amplification product = 532 bp

PCR conditions: Denaturation temperature = 94 °C for 1 min 30 sec

Annealing temperature = 53 °C for 1 min 30 sec

Extension temperature = 72 °C for 2 min

Cycles = 30

C) Transgenic poplar expressing the copper-controllable gene expression control-construct, pMB705

PCR primers were designed based on the *nptII* and provided by Dr. R. Meilan, Oregon State University. The forward and reverse primers used are as follows:

Forward : 5' TTCGTCCAGATCATCCTG 3' (18-mer)

Reverse : 5' TTCTTTTTGTCAAGACCG 3' (18-mer)

Expected amplification product = 343 bp

PCR conditions: Denaturation temperature = 94 °C for 1 min 30 sec

Annealing temperature = 38 °C for 1 min 30 sec

Extension temperature = 72 °C for 2 min

Cycles = 40

APPENDIX B: TRANSGENIC LINE IDENTIFICATION NUMBERS

Line number was the original number given to the kanamycin-resistant and PCR-verified transformants from tissue culture. Tree I.D. was given later for easy identification of individual lines. The asteriks (*) indicate negative *bO* expression, determined later through northern analysis.

Clone 189-434		Clone 195-529		Clone 311-93	
Line No.	Tree I.D.	Line No.	Tree I.D.	Line No.	Tree I.D.
16	16	15-3	1	19-12	29
18-2	17	30-1	2	43-2	31
21-1	19	44-1	3 *	6-1	32
22	20	20-1	4 *	14-1	33
19	21	14-1	5	44-1	35 *
25-1	22	18-3	6	62-3	36
20-2	23	28-3	7	63	37
32	24	25-4	8	52-2	38
33-1	25	6-6	10	Control	C3
36	26	1-2	11		
14-5	27	2-5	12		
35-1	28	4-5	13		
Control	C1	33-1	41		
		35-1	42		
		26-7	43		
		27-4	44		
		48-1	45		
		51-1	47		
		7-3	died		
		Control	C2		
Total number of transgenics	12		18		8

Table B.1 Transgenic poplar expressing the *bO* gene.

APPENDIX C: SPONTANEOUS LESION AND DISEASE ASESSMENT - LESION DEVELOPMENT AND RESISTANCE OF TRANSGENIC POPLARS TO *VENTURIA* LEAF AND SHOOT BLIGHT

Spontaneous lesion severity was rated as described in Materials and Methods (Chapter 2). Total lesion density (TLD) for leaf numbers 2 to 7 was assessed for each ramet in the field, and the score is reported as mean TLD determined from three ramets at each site. The leaf and shoot blight symptoms were rated as described in Materials and Methods (Chapter 2), on a categorical scale from 0-100 (100 = highly susceptible).

One SE of the mean from three ramets is shown above bars. Bars without standard errors indicate that all three ramets gave the same severity score, thus no SE could be calculated. Trans = transgenic; Cont = control (non-transgenic).

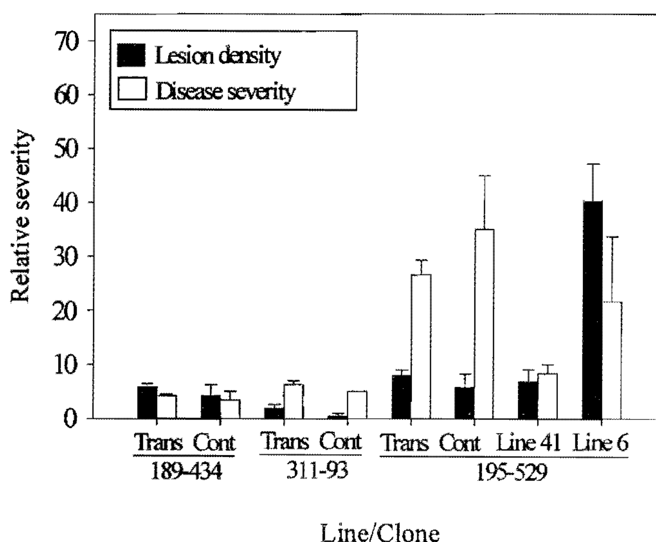

Figure C.1 Lesion density and disease severity at Peavy Arboretum.

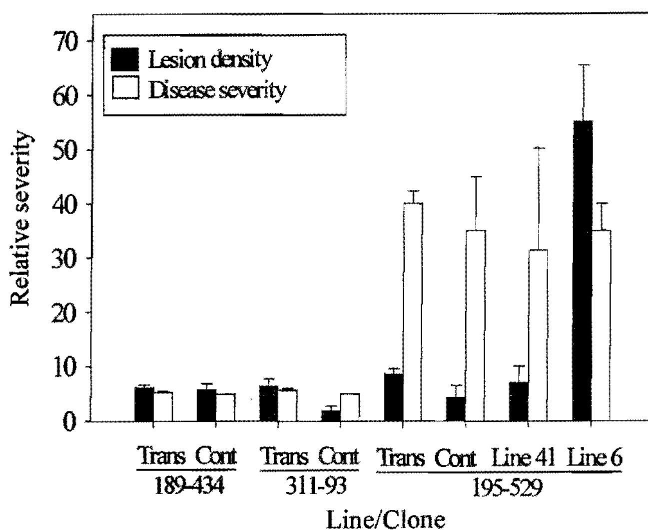

Figure C.2 Lesion density and disease severity at Marchel Tract.

Clone/Transgenic line		Peavy Arboretum		Marchel Tract		Both Sites		Transgenics and controls	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
189-434	Trans	5.7	0.67	6.1	0.55	5.9	0.44	5.9	0.41
189-434	Control	4.2	2.00	5.8	1.07	5.0	1.13		
311-93	Trans	1.8	0.72	6.4	1.36	3.9	0.72	3.7	0.68
311-93	Control	0.5	0.46	1.9	0.86	1.2	0.49		
195-529	Trans	8.0	1.02	8.6	0.98	8.3	0.71	8.1	0.68
195-529	Control	5.7	2.58	4.3	2.24	5.0	1.69		
Line 41		6.8	2.24	7.0	3.02	6.9	1.85		
Line 6		40.3	6.85	55.1	10.35	46.2	5.88		
All lines and controls over both sites		5.8	0.53	7.1	0.53				

Table C.1 Mean and SE of total lesion density (relative units).

Clone/Transgenic line		Peavy Arboretum		Marchel Tract		Both Sites		Transgenics and controls	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
189-434	Trans	4.1	0.31	5.3	0.19	4.7	0.20	4.7	0.19
189-434	Control	3.3	1.7	5.0	0	4.2	1.18		
311-93	Trans	6.3	0.68	5.7	0.36	6.0	0.43	5.8	0.35
311-93	Control	5.0	0	5.0	0	5.0	0		
195-529	Trans	26.8	2.70	40.1	2.25	33.3	2.04	33.0	1.79
195-529	Control	35.0	10.00	35.0	10.00	35.0	8.94		
Line 41		8.3	1.67	31.3	18.89	19.8	9.92		
Line 6		21.7	12.02	35.0	5.00	28.3	6.54		
All lines and controls over both sites		15.2	1.57	21.3	1.86				

Table C.2 Mean and SE of disease severity (relative units)

A) Lesion density

Statistical analysis

Analysis on lesion density was performed using the MIXED procedure, SAS (1990). The output is shown below. Data analyzed were lesion density from leaves 2 to 7 for each ramet, with a total of 6 ramets per line. A natural logarithm transformation was used to stabilize variance.

Tests of Fixed Effects

Source	NDF	DDF	Type III F	Pr > F
CLONE	2	2	8.07	0.1102
LINE(CLONE)	38	38	5.09	**0.0001**
LEAF	5	205	8.47	**0.0001**
CLONE*LEAF	10	205	6.06	**0.0001**
LINE*LEAF(CLONE)	190	205	0.92	0.7151

Least-squares Means

There was an interaction effect between clone and leaf number as discussed in conclusions (see below).

Effect	CLONE	LEAF	LSMEAN	SE	DF	t	Pr >|t|
CLONE*LEAF	189-434	2	-0.000	0.014	205	-0.00	1.0000
CLONE*LEAF	189-434	3	-0.000	0.014	205	-0.00	1.0000
CLONE*LEAF	189-434	4	0.085	0.014	205	6.18	0.0001
CLONE*LEAF	189-434	5	0.082	0.014	205	5.95	0.0001
CLONE*LEAF	189-434	6	0.090	0.014	205	6.52	0.0001
CLONE*LEAF	189-434	7	0.065	0.014	205	4.70	0.0001
CLONE*LEAF	195-529	2	0.025	0.012	205	2.11	0.0364
CLONE*LEAF	195-529	3	0.094	0.012	205	7.86	0.0001
CLONE*LEAF	195-529	4	0.084	0.012	205	7.00	0.0001
CLONE*LEAF	195-529	5	0.056	0.012	205	4.68	0.0001
CLONE*LEAF	195-529	6	0.076	0.012	205	6.36	0.0001
CLONE*LEAF	195-529	7	0.087	0.012	205	7.29	0.0001
CLONE*LEAF	311-93	2	0.030	0.016	205	1.87	0.0636
CLONE*LEAF	311-93	3	0.041	0.016	205	2.56	0.0111
CLONE*LEAF	311-93	4	0.033	0.016	205	2.05	0.0420
CLONE*LEAF	311-93	5	0.026	0.016	205	1.61	0.1086
CLONE*LEAF	311-93	6	0.035	0.016	205	2.22	0.0272
CLONE*LEAF	311-93	7	0.028	0.016	205	1.78	0.0765

Differences of Least-squares Means (at significance level of 0.05)

For the effect of clone, comparisons are between clone 1 (column 2) and clone 2 (column 4). For the effect of line within clone, comparisons are between transgenic lines (columns 2 and 3) against controls (columns 4 and 5). C1, C2 and C3 are the controls for clones 189-434, 195-529 and 311-93, respectively. The moderate (line 41) and high (line 6) *bO*-expressing lines are shown in bold.

1	2	3	4	5	6	7	8	9	10	11
Effect	CLONE	LINE	CLONE	LINE	Diff	SE	DF	t	Adjustment	Adj P
CLONE	189-434		195-529		-0.016	0.008	2	-1.98	Tukey-Kramer	0.3185
CLONE	189-434		311-93		0.021	0.010	2	2.07	Tukey-Kramer	0.2983
CLONE	195-529		311-93		0.038	0.009	2	3.98	Tukey-Kramer	0.1035
LINE(CLONE)	189-434	16	189-434	C1	0.008	0.033	38	0.26	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	17	189-434	C1	-0.022	0.033	38	-0.68	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	19	189-434	C1	0.011	0.033	38	0.34	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	20	189-434	C1	-0.013	0.033	38	-0.39	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	21	189-434	C1	0.025	0.033	38	0.75	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	22	189-434	C1	-0.012	0.033	38	-0.37	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	23	189-434	C1	0.027	0.033	38	0.82	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	24	189-434	C1	0.058	0.033	38	1.73	Dunnett-Hsu	0.7568
LINE(CLONE)	189-434	25	189-434	C1	0.021	0.033	38	0.64	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	26	189-434	C1	-0.007	0.033	38	-0.22	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	27	189-434	C1	0.020	0.033	38	0.62	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	28	189-434	C1	-0.028	0.033	38	-0.85	Dunnett-Hsu	0.9999
LINE(CLONE)	195-529	1	195-529	C2	0.026	0.033	38	0.79	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	10	195-529	C2	-0.026	0.033	38	-0.77	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	11	195-529	C2	-0.001	0.033	38	-0.05	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	12	195-529	C2	0.051	0.033	38	1.52	Dunnett-Hsu	0.8862
LINE(CLONE)	195-529	13	195-529	C2	0.000	0.033	38	0.00	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	2	195-529	C2	0.025	0.033	38	0.74	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	3	195-529	C2	0.018	0.033	38	0.55	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	4	195-529	C2	0.031	0.033	38	0.92	Dunnett-Hsu	0.9997
LINE(CLONE)	**195-529**	**41**	**195-529**	**C2**	**0.017**	**0.033**	**38**	**0.51**	**Dunnett-Hsu**	**1.0000**
LINE(CLONE)	195-529	42	195-529	C2	0.017	0.033	38	0.53	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	43	195-529	C2	-0.023	0.033	38	-0.70	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	44	195-529	C2	0.010	0.033	38	0.32	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	45	195-529	C2	0.010	0.033	38	0.32	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	47	195-529	C2	-0.007	0.033	38	-0.22	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	5	195-529	C2	-0.002	0.033	38	-0.09	Dunnett-Hsu	1.0000
LINE(CLONE)	**195-529**	**6**	**195-529**	**C2**	**0.320**	**0.033**	**38**	**9.48**	**Dunnett-Hsu**	**0.0000**
LINE(CLONE)	195-529	7	195-529	C2	0.012	0.033	38	0.36	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	8	195-529	C2	0.011	0.033	38	0.33	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	29	311-93	C3	0.019	0.033	38	0.59	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	31	311-93	C3	0.021	0.033	38	0.63	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	32	311-93	C3	0.022	0.033	38	0.68	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	33	311-93	C3	0.084	0.033	38	2.50	Dunnett-Hsu	0.2505
LINE(CLONE)	311-93	35	311-93	C3	0.020	0.033	38	0.59	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	36	311-93	C3	0.024	0.033	38	0.73	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	37	311-93	C3	-0.006	0.033	38	-0.20	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	38	311-93	C3	0.000	0.033	38	0.02	Dunnett-Hsu	1.0000

Conclusions:

1) There was no difference in mean TLD among the three clones ($p < 0.1102$). However, mean TLD from at least one line differed significantly within a particular clone ($p < 0.0001$); line 6 had a higher lesion density than control C2 (clone 195-529) ($p < 0.0000$, from Dunnett's test). The other lines did not differ significantly from their controls.

2) Lesion density differed significantly among leaf numbers 2 to 7 ($p < 0.0001$). There was no interaction between lesion density for line within a clone and leaf position ($p < 0.7151$). However, clone and leaf position interaction had a significant effect on lesion density ($p < 0.0001$). From LSM analysis of clone 189-434, leaf numbers 4 to 7 had more lesions compared to leaf 2 and 3. In clone 195-529, lesions from leaves 6 and 7 had a higher total lesion density if compared to leaf 2 and 3, or leaf 4 and 5. Finally, no obvious differences in lesion density were detected among the leaf positions in clone 311-93.

B) *Venturia* blight severity

Statistical analysis

Analysis on disease severity was performed using the GLM procedure, SAS (1990). The ANOVA table is shown below. Data analyzed were scores of disease severity rated on trees grown at both sites (6 ramets per line).

Source of Variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	81	64500.7	796.3	5.24	0.0001
Site	1	1354.7	1354.7	8.92	**0.0033**
Clone	2	47645.4	23822.7	17.61	**0.0537**
Site*Clone	2	2704.8	1352.4	8.90	0.0002
Line (Clone)	38	7958.4	209.4	1.38	**0.0335**
Site*Line (Clone)	38	4355.8	114.6	0.75	0.0469
Error	162	24606.7	151.9		
Corrected Total	243	89107.4			

Table C.3 ANOVA table for *Venturia* blight severity

Error terms used to construct F ratio:
1) Mean square of Site*Clone to test effect of Clone
2) Mean square of Site*Line(Clone) to test the effect of Line(Clone)
3) Mean square of Error to test the effect of Site, Site*Clone and Site*Line(Clone)

Duncan's Multiple Range Test

Alpha= 0.05 df= 158 MSE= 0.868707

Means with the same letter are not significantly different.

Duncan Grouping	*Mean*	*SE*	*N*	*SITE*
A	21.3	1.6	121	Marchel Tract
B	15.2	1.9	123	Peavy Arboretum

Duncan Grouping	*Mean*	*SE*	*N*	*CLONE*
A	33.0	1.8	113	195-529
B	5.9	0.4	53	311-93
B	4.7	0.2	78	189-434

Differences of Least-squares Means (at significance level of 0.05)

For the effect of clone, comparisons are between clone 1 (column 2) and clone 2 (column 4). For the effect of line within clone, comparisons are between transgenic lines (columns 2 and 3) against controls (columns 4 and 5). C1, C2 and C3 are the controls for clones 189-434, 195-529 and 311-93, respectively. The moderate (line 41) and high (line 6) *bO*-expressing lines are shown in bold.

1	2	3	4	5	6	7	8	9	10	11
Effect	CLONE	LINE	CLONE	LINE	Diff	SE	DF	t	Adjustment	Adj P
CLONE	189-434		195-529		28.481	5.642	2	-5.05	Tukey-Kramer	0.0669
CLONE	189-434		311-93		-1.168	5.770	2	-0.20	Tukey-Kramer	0.9779
CLONE	195-529		311-93		27.313	5.720	2	4.77	Tukey-Kramer	0.0742
LINE(CLONE)	189-434	16	189-434	C1	-0.000	6.945	200	-0.00	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	17	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	19	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	20	189-434	C1	-0.000	6.945	200	-0.00	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	21	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	22	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	23	189-434	C1	1.666	6.945	200	0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	24	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	25	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	26	189-434	C1	-1.666	6.945	200	-0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	27	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	28	189-434	C1	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	1	195-529	C2	-3.333	6.945	200	-0.48	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	10	195-529	C2	15.666	6.945	200	2.26	Dunnett-Hsu	0.3777
LINE(CLONE)	195-529	11	195-529	C2	15.666	6.945	200	2.26	Dunnett-Hsu	0.3777
LINE(CLONE)	195-529	12	195-529	C2	-1.666	6.945	200	-0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	13	195-529	C2	-0.166	6.945	200	-0.02	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	2	195-529	C2	-1.666	6.945	200	-0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	3	195-529	C2	6.666	6.945	200	0.96	Dunnett-Hsu	0.9999
LINE(CLONE)	195-529	4	195-529	C2	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	**195-529**	**41**	**195-529**	**C2**	**-8.500**	**6.945**	**200**	**-1.22**	**Dunnett-Hsu**	**0.9929**
LINE(CLONE)	195-529	42	195-529	C2	19.666	6.945	200	2.83	Dunnett-Hsu	0.1103
LINE(CLONE)	195-529	43	195-529	C2	1.666	6.945	200	0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	44	195-529	C2	5.833	6.945	200	0.84	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	45	195-529	C2	-5.000	6.945	200	-0.72	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	47	195-529	C2	16.729	7.288	200	2.30	Dunnett-Hsu	0.3514
LINE(CLONE)	195-529	5	195-529	C2	10.666	6.945	200	1.54	Dunnett-Hsu	0.9095
LINE(CLONE)	**195-529**	**6**	**195-529**	**C2**	**-0.000**	**6.945**	**200**	**-0.00**	**Dunnett-Hsu**	**1.0000**

LINE(CLONE)	195-529	7	195-529	C2	1.666	6.945	200	0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	8	195-529	C2	17.000	6.945	200	2.45	Dunnett-Hsu	0.2620
LINE(CLONE)	311-93	29	311-93	C3	1.666	6.945	200	0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	31	311-93	C3	1.666	6.945	200	0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	32	311-93	C3	0.833	6.945	200	0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	33	311-93	C3	-1.666	6.945	200	-0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	35	311-93	C3	0.965	7.291	200	0.13	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	36	311-93	C3	0.000	6.945	200	0.00	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	37	311-93	C3	2.500	6.945	200	0.36	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	38	311-93	C3	1.666	6.945	200	0.24	Dunnett-Hsu	1.0000

Conclusions:

1) There was convincing evidence that the disease was more severe at one site than the other ($p < 0.0033$). *Venturia* symptoms on the trees at MT were 1.4 times more severe than those trees at PA.

2) There was evidence that the clones performed differently against *Venturia* ($p < 0.0537$ for a test of clone effect). The Duncan's multiple range test grouped clone 195-529 separately from clones 189-434 and 311-93 (α=0.05). Clone 195-529 was more susceptible to *Venturia* and showed severe symptoms in the field. This clone displayed about 5.6 and 7 times more severe symptoms than clones 311-93 and 189-434, respectively.

3) There was evidence that at least one line performed differently from other lines within a clone ($p < 0.0335$ for a test of line within clone effect). However, there was no statistical evidence that any of the transgenics performed differently from its control as tested using the Dunnett's test (α=0.05), including the moderate (line 41) and high (line 6) *bO*-expressing lines.

APPENDIX D: ANALYSIS OF GROWTH RATE OF TRANSGENIC TREES IN FIELD TRIALS

Growth data was obtained by taking height and diameter of trees at PA and MT over one growing season, as described in Materials and Methods, Chapter 2. The mean net growth is displayed for all transgenic lines and the control for each clone by location, and by combining data over locations. Growth of the moderate (line 41) and high (line 6) *bO* expressing lines from clone 195-529 are shown separately. One SE of the mean is shown above bars. Statistical analysis was performed using the GLM procedure. The ANOVA table is shown below. Table D.4 consists of data from all the transgenic lines and the control, while Table D.5 excludes data from the high *bO* expression line (line 6).

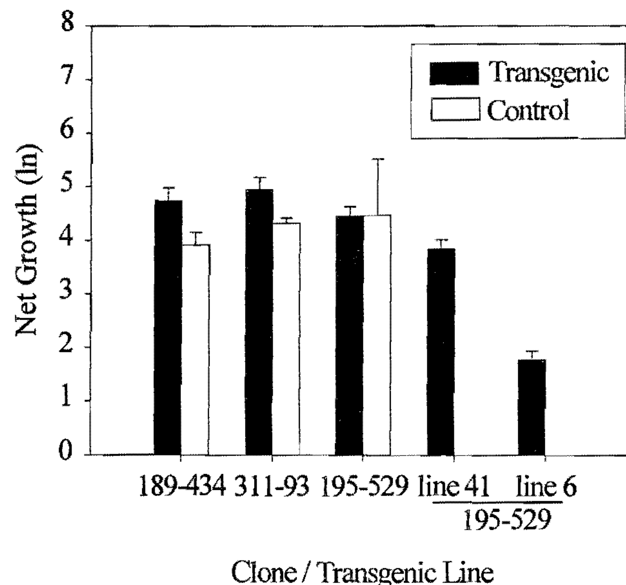

Figure D.1 Net growth at Peavy Arboretum.

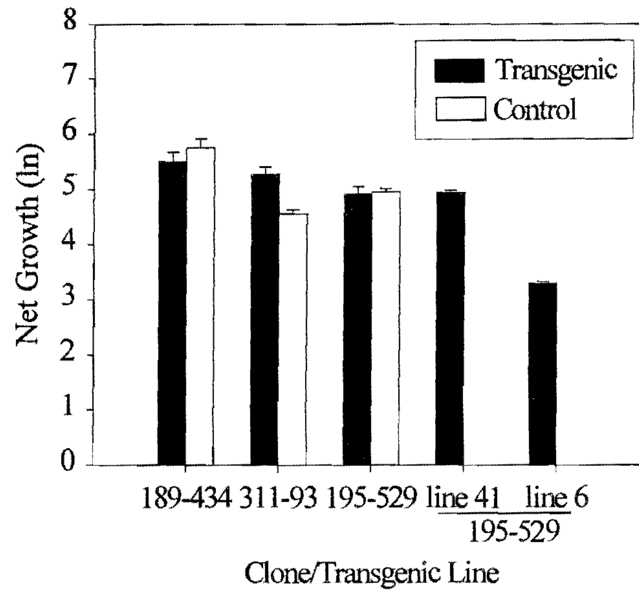

Figure D.2 Net growth at Marchel Tract.

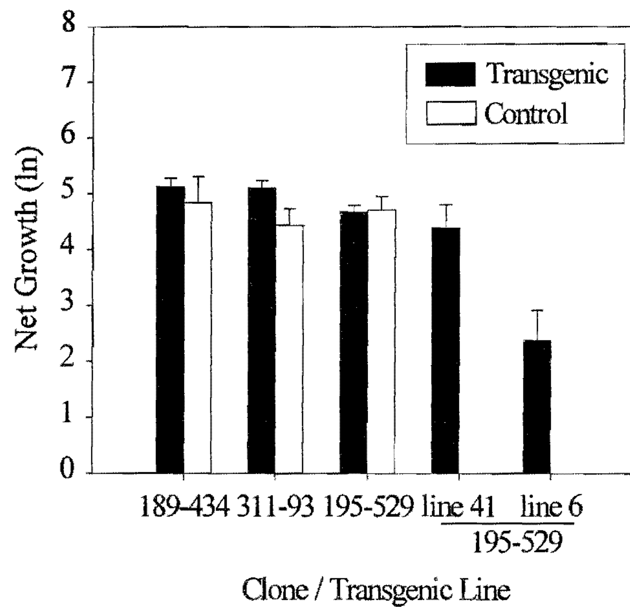

Figure D.3 Net growth over both sites.

Clone/Transgenic line		Peavy Arboretum		Marchel Tract		Both Sites		Transgenics and controls	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
189-434	Trans	4.7	0.24	5.51	0.17	5.13	0.15	5.1	0.16
189-434	Control	3.9	0.16	5.75	0.16	4.83	0.47		
311-93	Trans	4.9	0.23	5.27	0.14	5.10	0.13	5.0	0.13
311-93	Control	4.3	0.09	4.55	0.08	4.44	0.29		
195-529	Trans	4.4	0.17	4.91	0.14	4.67	0.11	4.7	0.10
195-529	Control	4.4	1.05	4.95	0.06	4.71	0.24		
Line 41		3.8	0.17	4.94	0.04	4.39	0.41		
Line 6		1.8	0.16	3.30	0.03	2.38	0.54		
All lines and controls over both sites		4.6	0.11	5.2	0.1				

Table D.1 Mean and SE of net growth in log units [(lnVI2) – (lnVI1)].

Multiplicative treatment effect after a log transformation in a randomized experiment:

1) Difference in mean net growth between PA and MT :
   $= 5.2 - 4.6 = 0.6$; $e^{0.6} = 1.82$
2) Difference in mean net growth between line 6 and control from clone 195-529 :
   $= 4.71 - 2.38 = 2.33$; $e^{2.33} = 10.3$

Clone/Transgenic line		Peavy Arboretum		Marchel Tract		Both Sites	
		Mean	SE	Mean	SE	Mean	SE
189-434	Trans	7	0.9	1124	148.8	573	100.3
189-434	Control	5	1.2	2909	865.2	1457	755.8
311-93	Trans	8	0.9	1250	272.7	616	160.6
311-93	Control	9	4.7	1825	642.7	917	497.5
195-529	Trans	9	1.0	1667	217.0	742	124.7
195-529	Control	8	5.1	2341	1055.6	1174	703.6
Line 41		5.9	3.3	476	18.6	241	105.5
Line 6		0.2	0.1	28	18.7	15	10.7
All lines and controls over both sites		8.0	0.6	1416	121	-	-

Table D.2 Mean and SE of net growth in 100 cm^3 (VI2-VI1).

Table D.3 Mean and SE of net growth (ln) for 3 ramets from each line and the controls at Peavy Arboretum and Marchel Tract.

Peavy Arboretum				Marchel Tract			
Clone	Line	Mean	SE	Clone	Line	Mean	SE
189-434	16	5.8	0.75	189-434	16	5.2	0.30
	17	5.3	0.25		17	5.8	0.81
	19	3.3	1.31		19	5.9	0.13
	20	4.3	0.29		20	6.0	0.51
	21	4.9	0.34		21	6.2	0.55
	22	4.4	0.59		22	5.1	0.16
	23	3.5	0.81		23	5.5	0.69
	24	4.3	0.17		24	3.3	0.16
	25	4.4	0.46		25	5.0	0.31
	26	6.5	1.14		26	6.1	0.29
	27	6.6	1.00		27	6.4	0.66
	28	4.2	0.45		28	5.5	0.37
	C1	3.9	0.16		C1	5.8	0.48
195-529	1	3.0	0.64	195-529	1	5.3	0.30
	2	4.5	0.73		2	4.9	0.80
	3	4.6	0.46		3	4.4	0.82
	4	5.1	0.48		4	4.7	0.08
	5	4.1	0.50		5	4.8	0.29
	6	1.8	0.69		6	3.3	0.24
	7	5.0	0.40		7	5.6	0.86
	8	4.1	0.78		8	4.3	0.26
	10	4.5	0.42		10	5.2	0.28
	11	5.3	0.11		11	5.2	0.51
	12	6.5	0.19		12	5.4	0.68
	13	4.7	0.35		13	4.9	0.82
	41	3.8	0.66		41	4.9	0.32
	42	4.9	0.18		42	5.3	0.61
	43	4.7	0.40		43	3.5	0.64
	44	5.1	1.05		44	5.8	0.55
	45	3.7	0.56		45	4.7	0.18
	47	4.7	0.32		47	6.5	0.41
	C2	4.5	0.37		C2	5.0	0.31
311-93	29	5.2	0.33	311-93	29	5.2	0.15
	31	5.0	0.42		31	5.5	0.35
	32	5.7	0.00		32	5.5	0.34
	33	5.0	0.04		33	4.8	0.35
	35	4.8	0.89		35	6.1	0.29
	36	4.9	0.14		36	5.3	0.47

Peavy Arboretum				Marchel Tract			
Clone	Line	Mean	SE	Clone	Line	Mean	SE
	37	3.4	1.02		37	5.4	0.70
	38	5.7	0.87		38	4.7	0.24
	C3	4.3	0.39		C3	4.5	0.52

Statistical analysis for growth

Source of Variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	81	180.2	2.22	2.56	0.0001
Site	1	16.2	16.17	18.62	**0.0001**
Clone	2	9.6	4.78	4.79	**0.1727**
Site*Clone	2	2.0	1.00	1.15	**0.3195**
Line (Clone)	38	98.1	2.58	1.99	**0.0183**
Site*Line (Clone)	38	49.3	1.30	1.49	0.0469
Error	158	137.3	0.87		
Corrected Total	239	317.5			

Table D.4 ANOVA table for net growth.

Source of Variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	79	145.1	1.84	2.12	0.0001
Site	1	14.8	14.84	17.13	**0.0001**
Clone	2	4.7	2.33	2.04	**0.3219**
Site*Clone	2	2.3	1.14	1.32	**0.2707**
Line (Clone)	37	74.4	2.01	1.56	**0.0911**
Site*Line (Clone)	37	47.7	1.29	1.49	0.0498
Error	155	134.3	0.87		
Corrected Total	234	279.4			

Table D.5 ANOVA table for net growth (after removing data from line 6).

Error terms used to construct F ratio:

1) Mean square of Site*Clone to test effect of Clone
2) Mean square of Site*Line(Clone) to test the effect of Line(Clone)
3) Mean square of Error to test the effect of Site, Site*Clone and Site*Line(Clone)

Duncan's Multiple Range Test

Alpha= 0.05 df= 158 MSE= 0.868707

Means with the same letter are not significantly different.

Duncan Grouping	*Mean*	*SE*	*N*	*SITE*
A	5.2	0.1	118	Marchel Tract
B	4.6	0.1	122	Peavy Arboretum

Differences of Least-squares Means (at significance level of 0.05)

For the effect of clone, comparisons are between clone one (column 2) to clone 2 (column 4). For the effect of line within clone, comparisons are between transgenic lines (columns 2 and 3) against controls (columns 4 and 5). C1, C2 and C3 are the controls for clones 189-434, 195-529 and 311-93, respectively. The moderate (line 41) and high (line 6) *bO*-expressing lines are shown in bold.

1	2	3	4	5	6	7	8	9	10	11
Effect	CLONE	LINE	CLONE	LINE	Diff	SE	DF	t	Adjustment	Adj P
CLONE	189-434		195-529		0.445	0.170	2	2.61	Tukey-Kramer	0.2115
CLONE	189-434		311-93		0.077	0.196	2	0.39	Tukey-Kramer	0.9212
CLONE	195-529		311-93		-0.368	0.186	2	-1.97	Tukey-Kramer	0.3188
LINE(CLONE)	189-434	16	189-434	C1	0.641	0.563	196	1.14	Dunnett-Hsu	0.9969
LINE(CLONE)	189-434	17	189-434	C1	0.675	0.563	196	1.20	Dunnett-Hsu	0.9933
LINE(CLONE)	189-434	19	189-434	C1	-0.248	0.563	196	-0.44	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	20	189-434	C1	0.349	0.563	196	0.62	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	21	189-434	C1	0.715	0.563	196	1.27	Dunnett-Hsu	0.9855
LINE(CLONE)	189-434	22	189-434	C1	-0.068	0.563	196	-0.12	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	23	189-434	C1	-0.370	0.563	196	-0.66	Dunnett-Hsu	1.0000

LINE(CLONE)	189-434	24	189-434	C1	-1.012	0.563	196	-1.80	Dunnett-Hsu	0.7135
LINE(CLONE)	189-434	25	189-434	C1	-0.107	0.563	196	-0.19	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	26	189-434	C1	1.359	0.590	196	2.30	Dunnett-Hsu	0.3338
LINE(CLONE)	189-434	27	189-434	C1	1.699	0.563	196	3.02	Dunnett-Hsu	0.0660
LINE(CLONE)	189-434	28	189-434	C1	0.014	0.563	196	0.03	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	1	189-434	C1	-0.854	0.597	196	-1.43	Dunnett-Hsu	0.9449
LINE(CLONE)	195-529	10	189-434	C1	0.044	0.570	196	0.08	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	11	189-434	C1	0.439	0.570	196	0.77	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	12	189-434	C1	1.108	0.570	196	1.94	Dunnett-Hsu	0.5941
LINE(CLONE)	195-529	13	189-434	C1	-0.064	0.570	196	-0.11	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	2	195-529	C2	0.019	0.563	196	0.03	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	3	195-529	C2	-0.245	0.563	196	-0.44	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	4	195-529	C2	0.163	0.563	196	0.29	Dunnett-Hsu	1.0000
LINE(CLONE)	**195-529**	**41**	**195-529**	**C2**	**-0.318**	**0.563**	**196**	**-0.57**	**Dunnett-Hsu**	**1.0000**
LINE(CLONE)	195-529	42	195-529	C2	0.383	0.563	196	0.68	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	43	195-529	C2	-0.611	0.563	196	-1.08	Dunnett-Hsu	0.9987
LINE(CLONE)	195-529	44	195-529	C2	0.771	0.563	196	1.37	Dunnett-Hsu	0.9651
LINE(CLONE)	195-529	45	195-529	C2	-0.498	0.563	196	-0.88	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	47	195-529	C2	0.739	0.590	196	1.25	Dunnett-Hsu	0.9882
LINE(CLONE)	195-529	5	195-529	C2	-0.247	0.563	196	-0.44	Dunnett-Hsu	1.0000
LINE(CLONE)	**195-529**	**6**	**195-529**	**C2**	**-2.284**	**0.590**	**196**	**-3.87**	**Dunnett-Hsu**	**0.0047**
LINE(CLONE)	195-529	7	195-529	C2	0.589	0.590	196	1.00	Dunnett-Hsu	0.9997
LINE(CLONE)	195-529	8	195-529	C2	-0.508	0.563	196	-0.90	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	29	311-93	C3	0.752	0.563	196	1.34	Dunnett-Hsu	0.9729
LINE(CLONE)	311-93	31	311-93	C3	0.771	0.563	196	1.37	Dunnett-Hsu	0.9645
LINE(CLONE)	311-93	32	311-93	C3	1.149	0.563	196	2.04	Dunnett-Hsu	0.5156
LINE(CLONE)	311-93	33	311-93	C3	0.452	0.563	196	0.80	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	35	311-93	C3	0.907	0.590	196	1.54	Dunnett-Hsu	0.8953
LINE(CLONE)	311-93	36	311-93	C3	0.623	0.563	196	1.11	Dunnett-Hsu	0.9980
LINE(CLONE)	311-93	37	311-93	C3	-0.025	0.563	196	-0.05	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	38	311-93	C3	0.782	0.563	196	1.39	Dunnett-Hsu	0.9583

Conclusions:

1) There was conclusive evidence that the trees at MT grew 1.82 times faster than PA ($p < 0.0001$ for a test of site effect). There was no evidence that the clones grew differently on either site ($p < 0.1727$ for a test of clone effect). No evidence of interaction between site and clone ($p < 0.3195$).

2) There was evidence that at least one line grew differently than the other lines in a single clone ($p < 0.0183$ for a test of line within clone effect). When data from this line (6, clone 195-529) was excluded, the line within clone effect became non-significant ($p < 0.0911$, see Table D.5). There was strong evidence that line 6 had retarded growth when compared to its control (C2) from clone 195-529 ($p < 0.0047$, Dunnett's test, $\alpha=0.05$); C2 grew 10.3 times faster than line 6. All other transgenic lines, including the moderate expressing line 41, were not significantly different in growth compared to their controls (Dunnett's test, $\alpha=0.05$).

APPENDIX E: QUANTIFICATION OF TRANSGENE AND *PAL1* EXPRESSION IN TRANSGENIC PLANTS

Two RNA blots (A and B), were prepared for running samples from all transgenic and control lines. Northern analysis was as described in Materials and Methods (Chapter 2). RNA blots were probed with the *bO* and *PAL1* genes. RNA samples for control plants (C1, C2 and C3) and transgenic line 17 were included in both blots. Expression from line 17 was used to standardize *bO* expression, after loading was corrected for all samples based on rDNA hybridization (its relative *bO* expression was set to 1.0).

Correlation analysis was performed using the GLM procedure, and the difference between transgenic and non-transgenic means was tested using the Student's *t*-test. Statistical analysis was performed using SAS (1990).

A) Expression of *PAL1* and *bO* from all transgenic and control lines

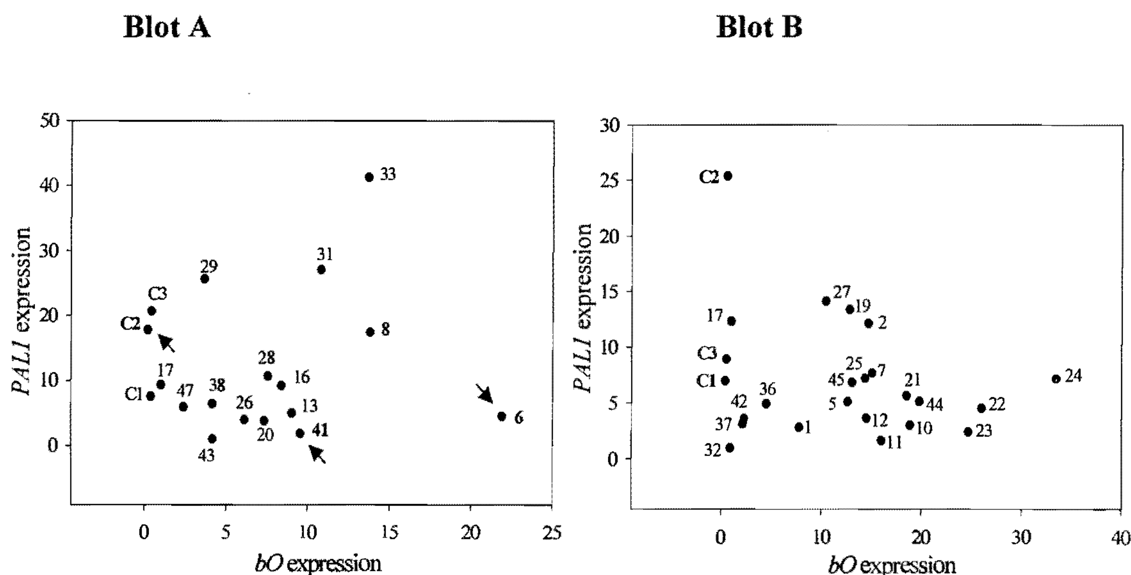

Figure E.1 Relationship between *PAL1* and *bO* expression in transgenic and control lines as quantified from RNA blot A (left) and blot B (right). Arrows are shown for lines 41 and 6, the moderate and high *bO*-expressing lines respectively, while C2 is the non-transgenic line for this clone.

Statistical analysis

Correlation analysis

Data set from	r	p-value
Blot A	-0.00150	0.9950
Blot B	-0.13154	0.5308
Blot A (minus outlier Line 6)	0.07895	0.7480
Blot A (Clone 195-529 only)	-0.26667	0.4879
Blot B (Clone 195-529 only)	-0.20000	0.5796

Table E.1 Correlation analysis for variable *PAL1* and *bO*. Data from blot A and blot B were analyzed separately. The Spearman correlation coefficients (r) are shown.

T-test

Comparing means for *PAL1* expression between transgenic and control groups. Output from the *t*-test is shown below. Data are from the means over 39 transgenic lines (including two analyses for line 17), and 3 controls (two analyses for each clone). Class TYPE: 1 = transgenic and 2 = control.

TYPE	N	Mean	SD	SE
1	39	8.27	8.18	1.31
2	6	14.57	7.76	3.17

Variances	T	Method	DF	Prob>|T|
Unequal	-1.84	Satterthwaite	6.8	0.1099
		Cochran	.	0.1189
Equal	-1.77		43.0	**0.0843**

For H_0: Variances are equal, F' = 1.11 DF = (38,5) Prob>F' = 1.00

The *t*-test, under the assumptions of equal variance, showed that the mean *PAL1* expression for transgenic and control groups was not significantly different ($p < 0.0843$).

Conclusions:

1) There was no correlation between the expression of *PAL1* and *bO* ($r = -0.00150$, $p < 0.9950$ for expression quantified from blot A, and $r = -0.13154$, $p < 0.5308$ from blot B). *PAL1* expression is not correlated with *bO* expression or vice versa.
2) There was no difference in the mean *PAL1* expression between transgenics and controls ($p < 0.0843$).

B) Relationship between TLD and *bO* expression

Statistical analysis

Correlation analysis for variable mean TLD and *bO* expression. Data are from all transgenic lines including the control from clone 195-529. Mean TLD was calculated for 6 trees grown at both field sites, and *bO* expression was quantified from the above two blots. The Pearson correlation coefficient for the two variables is 0.3170 ($p < 0.1860$).

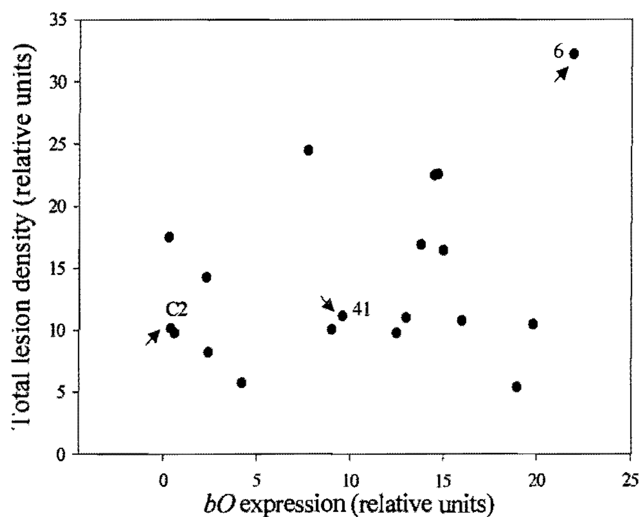

Figure E.2 Relationship between total lesion density and *bO* expression. Lines 41 and 6 are the moderate and high *bO*-expressing lines, respectively, while C2 is the non-transgenic.

Conclusions:

Although the effect of *bO* gene on lesion formation in line 6 was obvious, the relationship between lesion density and *bO* expression in clone 195-529 is not statistically significant ($r = 0.3170$, $p < 0.1860$).

APPENDIX F: EXPRESSION AND QUANTIFICATION OF DEFENSE-RELATED GENES IN TRANSGENIC PLANTS

Northern analysis of expression of *bO* and several defense related genes (*win6*, *win8* and *PAL1*) is shown in Figure F.1. The 18S rDNA hybridization was used to adjust for variation in RNA loading per lane. Transgenic lines 6, 41 and 12 were from clone 195-529, and represented high, moderate and low levels of HR-lesion intensity, from preliminary greenhouse observation. Wounding experiment design, RNA extraction, northern hybridization, and RNA quantification were as described in Materials and Methods (Chapter 2). RNA samples were collected from leaves of damaged (D) and undamaged (UD) trees, as well as from shoot apex (including the first expanding leaf).

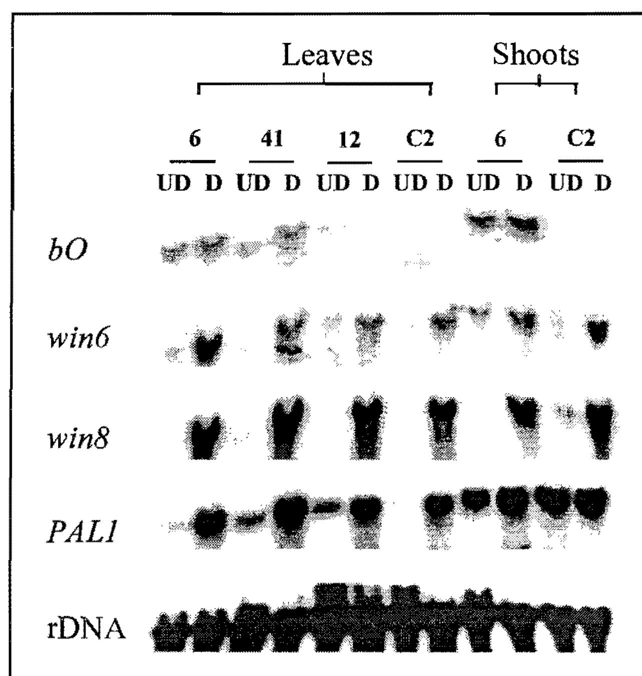

Figure F.1 Northern blot analysis of transgenic lines expressing *bO* and defense-related genes after induction via mechanical wounding.

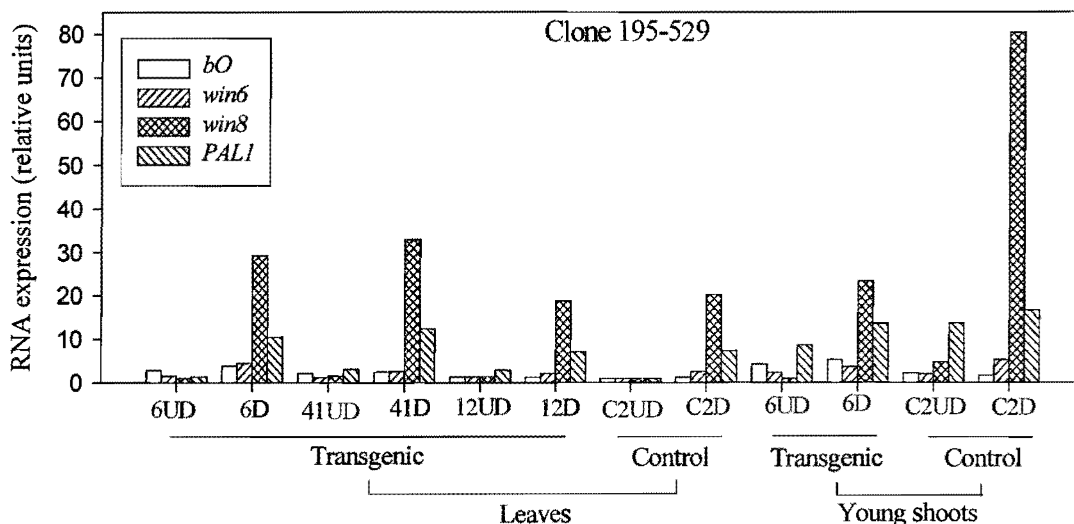

Figure F.2 RNA expression of several defense-related genes after induction via mechanical wounding. Expression was quantified from the above blot.

Line	Treatment	Tissue type	Gene expression (relative units)			
			bO	*win6*	*win8*	*PAL1*
6	UD	Leaf	2.8	1.6	1.0	1.3
6	D	Leaf	3.8	4.5	29.2	10.4
6	UD	Shoot	4.2	2.3	1.0	8.6
6	D	Shoot	5.2	3.6	23.3	13.6
41	UD	Leaf	2.2	1.2	1.6	3.1
41	D	Leaf	2.4	2.7	32.9	12.4
12	UD	Leaf	1.4	1.4	1.5	2.9
12	D	Leaf	1.3	2.2	18.7	7.1
C2	UD	Leaf	1.0	1.0	1.1	1.0
C2	D	Leaf	1.2	2.5	20.2	7.3
C2	UD	Shoot	2.1	1.9	4.6	13.6
C2	D	Shoot	1.5	5.2	80.2	16.5

Table F.1 RNA expression of several defense-related genes after induction via mechanical wounding as quantified from the above blot.

Undamaged tree			Damaged tree		
Gene	Mean	SE	Gene	Mean	SE
bO	1.8	0.40	*bO*	2.2	0.62
win6	1.3	0.12	*win 6*	3.0	0.53
win8	1.3	0.13	*win 8*	25.3	3.45
PAL1	2.1	0.54	*PAL1*	9.3	1.29

Table F.2 Mean and SE (relative unit) of gene expression. Data are from expression in the leaf samples of both transgenic and control plants, both undamaged and damaged.

Statistical analysis on the effect of wounding on gene expression

Statistical analysis was performed using the MIXED procedure, SAS (1990). The output is shown below. The variable was expression of different genes in the leaf tissues, from both transgenic and control plants, in each UD or D groups. Values were transformed by taking the natural logarithm, and the difference between expression under damaged and undamaged treatments (induced expression) was analyzed.

Tests of Fixed Effects

Source	NDF	DDF	Type III F	Pr > F
GENE	3	9	157.84	**0.0001**

Least-squares Means

LSM analysis of the difference in expression between undamaged and damaged groups (due to wounding).

Effect	GENE	LSMEAN	SE	DF	t	Pr > |t|
GENE	***Win8***	**2.964**	**0.177**	**9**	**16.71**	**0.0001**
GENE	***PAL1***	**1.578**	**0.177**	**9**	**8.90**	**0.0001**
GENE	***Win6***	**0.806**	**0.177**	**9**	**4.55**	**0.0014**
GENE	*bO*	0.128	0.177	9	0.73	0.4864

Conclusions:

1) There was strong evidence that wounding affected expression of at least one of the four genes ($p < 0.0001$).
2) LSM analysis showed that wounding increased the expression of *win8* ($p < 0.0001$), *PAL1* ($p < 0.0001$) and *win6* ($p < 0.0014$). However, the expression of *bO* was not affected by wounding ($p < 0.4864$).

APPENDIX G: DISEASE ASESSMENT - RESISTANCE OF TRANSGENIC POPLARS TO *MELAMPSORA* LEAF RUST

Selected transgenic lines and control used for rust inoculation study in the greenhouse:

Clone 189-434 : Tree I.D. number 16, 17, 20, 26 and 28, and control, C1

Clone 195-529 : Tree I.D. number 2, 3, 4, 6, 8, 13, 41, 43, and 47, and control, C2

Clone 311-93 : Tree I.D. number 29, 31, 33, 36 and 38, and control, C3

Rust severity was rated on a categorical scale from 0-100 (highly susceptible), as in Materials and Methods (Chapter 2).

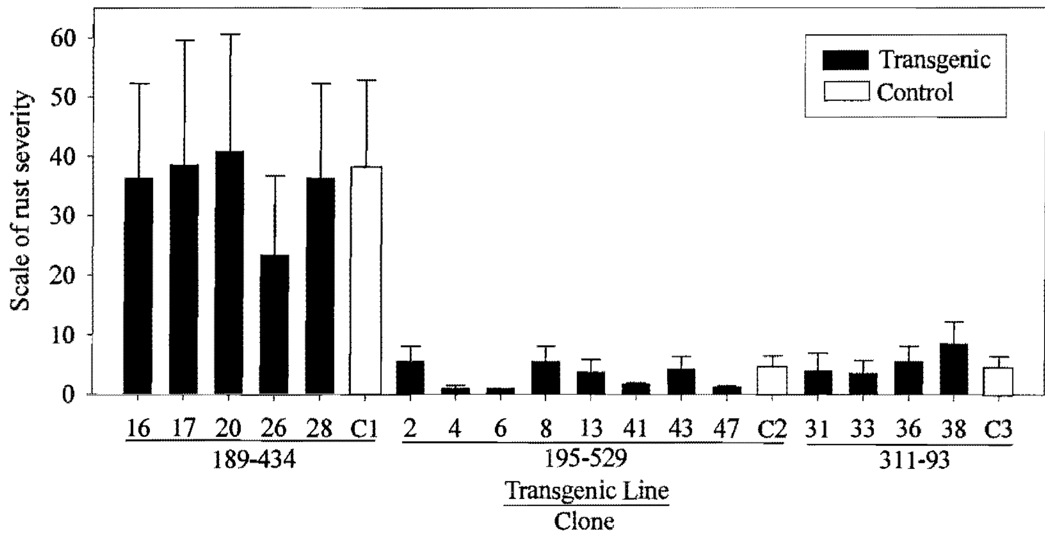

Figure G.1 *Melampsora* rust severity on transgenic lines and control plants. Data are the mean rust severity across four ramets from each transgenic and control plant. One SE of the mean is shown above bars. The bar without SE (line 6) is a result of there being no variation in the scores from all four ramets, thus SE could not be calculated.

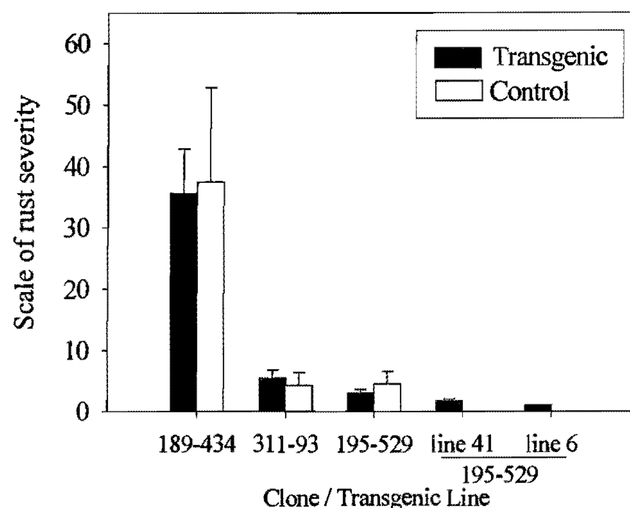

Figure G.2 Clonal means for rust severity. Data are the mean rust severity from all transgenic lines and control in each clone. One SE of the mean is shown above bars. Lines 41 and 6 are the moderate and high *bO*-expressing lines from clone 195-529.

Clone/Transgenic line		Mean	SE
189-434	Transgenic	35.6	7.22
189-434	Control	37.5	15.34
311-93	Transgenic	5.5	1.37
311-93	Control	4.3	2.15
195-529	Transgenic	3.0	0.59
195-529	Control	4.5	2.02
Line 41		1.8	0.25
Line 6		1.0	0.00

Table G.1 Mean and SE of rust severity (on a categorical scale of 0 - 100)

Statistical analysis

Analysis on disease severity was performed using the MIXED procedure, SAS (1990). The output is shown below. Data analyzed were scores for rust severity from 4 ramets of each transgenic line and control, after transformation by taking the natural logarithm.

Tests of Fixed Effects

Source	NDF	DDF	Type III F	Pr > F
CLONE	2	2	21.74	**0.0440**
LINE(CLONE)	17	17	0.84	0.6416

Least-squares Means

LSM analysis on rust severity that is due to clone type.

Effect	CLONE	LSMEAN	SE	DF	t	Pr > |t|
CLONE	**189-434**	**3.073**	**0.703**	**2**	**4.37**	**0.0487**
CLONE	195-529	1.148	0.698	2	1.64	0.2417
CLONE	311-93	1.524	0.707	2	2.15	0.1640

Differences of Least-squares Means (at significance level of 0.05)

For the effect of clone, comparisons are between clone 1 (column 2) to clone 2 (column 4). Clone 195-529 and 311-93 did not differ in their levels of resistance to rust (shown in bold). For the effect of line within clone, comparisons are between transgenic lines (columns 2 and 3) against controls (columns 4 and 5). C1, C2 and C3 are the controls for clones 189-434, 195-529 and 311-93, respectively.

1	2	3	4	5	6	7	8	9	10	11
Effect	CLONE	LINE	CLONE	LINE	Diff	SE	DF	t	Adjustment	Adj P
CLONE	189-434		195-529		1.924	0.303	2	6.34	Tukey-Kramer	0.0434
CLONE	189-434		311-93		1.549	0.323	2	4.78	Tukey-Kramer	0.0739
CLONE	**195-529**		**311-93**		**-0.375**	**0.311**	**2**	**-1.21**	**Tukey-Kramer**	**0.5555**
LINE(CLONE)	189-434	16	189-434	c1	-0.093	0.518	17	-0.18	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	17	189-434	c1	-0.643	0.518	17	-1.24	Dunnett-Hsu	0.9009
LINE(CLONE)	189-434	20	189-434	c1	-0.246	0.518	17	-0.48	Dunnett-Hsu	1.0000
LINE(CLONE)	189-434	26	189-434	c1	-0.632	0.536	17	-1.18	Dunnett-Hsu	0.9272
LINE(CLONE)	189-434	28	189-434	c1	-0.093	0.518	17	-0.18	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	13	195-529	c2	-0.173	0.518	17	-0.33	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	2	195-529	c2	0.050	0.518	17	0.10	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	4	195-529	c2	-0.946	0.518	17	-1.83	Dunnett-Hsu	0.5478
LINE(CLONE)	195-529	41	195-529	c2	-0.498	0.518	17	-0.96	Dunnett-Hsu	0.9845
LINE(CLONE)	195-529	43	195-529	c2	-0.101	0.518	17	-0.20	Dunnett-Hsu	1.0000
LINE(CLONE)	195-529	47	195-529	c2	-0.700	0.518	17	-1.35	Dunnett-Hsu	0.8532
LINE(CLONE)	195-529	6	195-529	c2	-0.802	0.518	17	-1.55	Dunnett-Hsu	0.7346
LINE(CLONE)	195-529	8	195-529	c2	0.050	0.518	17	0.10	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	31	311-93	c3	0.130	0.536	17	0.24	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	33	311-93	c3	-0.173	0.518	17	-0.33	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	36	311-93	c3	0.151	0.518	17	0.29	Dunnett-Hsu	1.0000
LINE(CLONE)	311-93	38	311-93	c3	0.541	0.518	17	1.05	Dunnett-Hsu	0.9663

Conclusions:

1) There was strong evidence that the three clones varied in their degree of resistance toward rust infection ($p < 0.0440$). Clones 195-529 and 311-93 were more resistant to rust than clone 189-434 (Tukey-Kramer's test, $\alpha=0.05$).
2) There was no evidence that lines within clones differed in their degree of resistance towards rust ($p < 0.6416$). Transgenic lines did not perform better than the controls against rust infection (Dunnett's test, $\alpha=0.05$).

APPENDIX H: RESISTANCE OF *bo* TRANSGENIC POPLARS TO *SEPTORIA* LEAF SPOT AND STEM CANKER (U.S. FOREST SERVICE)

Inter-nodal cuttings were made from 2-month old plants propagated in ½ MS medium. Cuttings were inserted into sterile culture tubes (Nalgene), partially filled with solid ½ MS medium and sent via overnight express parcel service to USFS North Central Research Station, St. Paul, MN. Plants were then propagated *in vitro* and transferred into soil in the greenhouse. *Septoria* assays were carried out by Dr. M.E. Ostry and Ms. K.T. Ward, using laboratory assays established in their lab. Three ramets from each of 36 transgenic and 3 control lines were grown for the assays. Leaf and stem assays and *in vitro* inoculation are as described in Materials and Methods (Chapter 2).

Clone 189-434		Clone 195-529		Clone 311-93	
USFS No.	OSU I.D.	USFS No.	OSU I.D.	USFS No.	OSU I.D.
7	17	25	1	16	29
9	19	33	2	17	31
3	20	37	3	20	32
10	22	28	4	15	33
8	23	24	5	18	35
4	24	26	6	21	36
11	25	32	7	14	37
5	26	29	8	19	38
6	27	40	10	Control	C3
Control	C1	23	11		
		27	12		
		36	13		
		34	41		
		35	42		
		30	43		
		31	44		
		38	45		
		39	47		
		41	7-3 (died)		
		Control	C2		
Total transgenics	9		19		8

Table H.1 Designation of transgenic and control lines. 'USFS No.' is the designation given by the Forest Service. 'OSU I.D.' is the reference number used in Appendix B.

Tree I.D.	Leaf Replicates	Leaf Assay Green tissue (%)		Stem Assay (12 weeks) Canker area (cm^2)	
Clone 189-434		*S. musiva*	*S. populicola*	*S. musiva*	*S. populicola*
20	36	3	20	7.7	6.8
24	54	2	19	7.5	4.0
26	54	3	16	10.0	4.4
27	54	4	16	8.2	6.8
17	54	3	14	12.3	4.8
23	54	1	19	13.9	9.7
19	54	4	12	5.2	5.6
22	36	8	16	5.4	6.5
25	36	1	11	13.3	11.0
Transgenic Mean		**3 ± 0.7**	**16 ± 2.0**	**9.3 ± 1.0**	**6.6 ± 0.8**
Control Mean	54	**1 ± 0.7**	**13 ± 10.5**	**9.5 ± 1.9**	**5.1 ± 1.0**

Table H.2 Screening for resistance toward *Septoria* leaf spot and stem canker in transgenic and control plants from clone 189-434.

Tree I.D.	Leaf Replicates	Leaf Assay Green tissue (%)		Stem Assay (12 weeks) Canker area (cm^2)	
Clone 311-93		*S. musiva*	*S. populicola*	*S. musiva*	*S. populicola*
37	36	16	22	2.5	11.1
33	36	8	21	1.9	11.4
29	36	13	17	1.8	10.3
31	36	8	14	3.1	5.6
35	36	9	18	8.0	11.8
38	36	13	14	4.0	5.3
32	36	9	25	4.2	5.6
36	24	3	11	10.5	8.9
Transgenic Mean		**10 ± 2.3**	**18 ± 1.7**	**4.5 ± 1.0**	**8.8 ± 0.9**
Control Mean	36	**4 ± 3.0**	**15 ± 4.5**	**2.6 ± 0**	**6.8 ± 2.6**

Table H.3 Screening for resistance toward *Septoria* leaf spot and stem canker in transgenic and control plants from clone 311-93.

Tree I.D.	Leaf Replicates	Leaf Assay Green tissue (%)		Stem Assay (12 weeks) Canker area (cm^2)	
Clone 195-529		*S. musiva*	*S. populicola*	*S. musiva*	*S. populicola*
11	36	16	30	8.9	5.1
5	54	11	31	12.6	4.4
1	54	7	12	11.0	6.5
6	54	3	19	5.7	5.8
12	54	13	32	7.8	8.5
4	54	5	26	10.7	5.7
8	54	6	28	4.5	4.0
43	54	15	15	6.9	3.1
44	54	37	56* R	7.5	8.0
7	54	28	44*	10.5	3.4
2	54	37	64* R	6.0	5.7
41	54	29	59* R	7.6	6.3
42	54	30	63* R	5.2	2.4
13	36	6	16	7.3	4.7
3	54	35	52* R	8.7	5.8
45	54	32	55* R	7.6	2.5
47	54	27	48*	4.1	5.6
10	54	31	59* R	7.9	9.2
7-3	54	17	38	5.7	3.9
Transgenic Mean		**20 ± 2.3**	**39 ± 3.3**	**7.7 ± 0.5**	**5.3 ± 0.5**
Control Mean	90	**31 ± 4.9**	**43 ± 9.5***	**6.8 ± 1.9**	**3.3 ± 0.5**

Table H.4 Screening for resistance toward *Septoria* leaf spot and stem canker in transgenic and control plants from clone 195-529.

	Leaf Assay Green tissue (%)		Stem Assay (12 weeks) Canker area (cm2)	
	S. musiva	*S. populicola*	*S. musiva*	*S. populicola*
All Transgenics Mean	**15 ± 1.6**	**31 ± 2.3**	**7 ± 0.5**	**6 ± 0.4**
All Control Mean	**17 ± 5.3**	**29 ± 7.2**	**6 ± 1.3**	**4 ± 0.5**
Line 41	**29 ± 3.6**	**59 ± 2.7**	**7.6 ± 1.1**	**6.3 ± 1.2**
Line 6	**3 ± 1.1**	**19 ± 4.3**	**5.7 ± 2.7**	**5.8 ± 1.9**

Table H.5 Clonal means for resistance toward *Septoria* leaf spot and stem canker. Included are data from the moderate (line 41) and high (line 6) *bO*-expressing lines.

*leaf displayed HR-like lesions, observed only during the first assay.
R = >50% healthy leaf tissue

APPENDIX I: SUMMARY : EFFECT OF WATER STRESS ON LESION FORMATION IN TRANSGENIC POPLARS (U.S. FOREST SERVICE)

This study was carried out by Sarah Netzer at the USFS, North Central Research Station, St. Paul, MN, with the assistance from USFS staffs, and supervised by Dr. C. Michler.

Objective

To study the ability of water stress at inducing lesions in *bO*-expressing poplars.

Method

We selected 6 transgenic lines from clone 195-529 for study. The lines selected included the high (line 6), medium (line 41) and low (lines 8, 13, 43, and 47) *bO*-expressers, and the control (C2). Cuttings (4 ramets per line) were grown in the greenhouse for 7 weeks, during which time all trees had reached an average leaf plastochron index (LPI) target of 24. Two ramets were well-watered (WW) and the other two were deprived of water (water-stressed, WS). The WS trees were watered only once or twice during the 16-day study period, depending on water status measured using a sensor (Time Domain Reflector), or when physical signs of water stress were apparent (leaves drooping). Data were collected on the first, fourth, ninth and sixteenth days during the study period. To quantify lesion formation, two leaves from each ramet were subjected to image analysis (Optimas computer software). Area covered by lesions on two leaves (selected between LPI 10 and LPI 14) from each ramet were monitored, and reported as the percentage (%) of total leaf area covered by lesions after 16 days. Data were analyzed using the mean % lesion area (leaf damage) over both leaves from two ramets. Lines

were ranked based on the leaf damage observed and analyzed using the Kruskal-Wallis test. Analysis of treatment effect on leaf damage was performed (after data transformation by taking the natural logarithm) using the Student's *t*-test via SAS (1990).

Results

The WS trees displayed more lesions than WW trees in all the lines tested except for line 41 (Figure I.1 and Table I.1); however, these differences were not significantly proven ($p < 0.79$, from Student's *t*-test). Although the mean % lesions appeared higher in WS trees (Table I.1), apart from line 6, their levels were extremely low: the mean leaf damage was less than 1%. We reanalyzed the data after removing leaf damage score from the high lesion-developing line (6), lesion area was still not significantly different between WS and WW trees ($p < 0.18$). Lesions were not observed on any of the control trees.

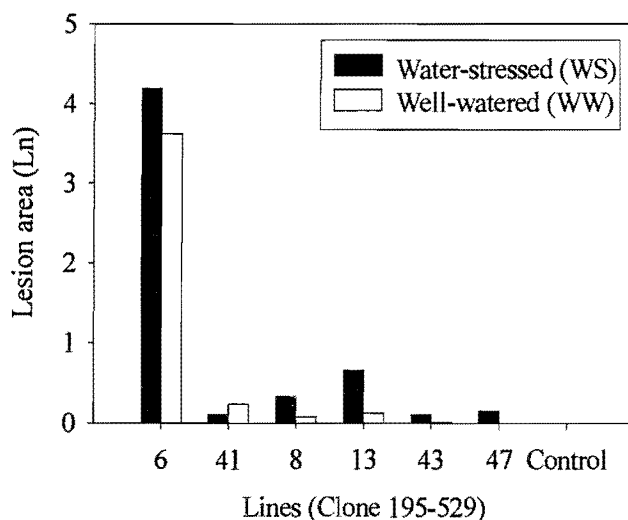

Figure I.1 Lesion development in trees grown under WS or WW conditions over a 16-day period. Data are mean % lesion area, after a natural logarithmic transformation, over four leaves from two ramets.

Lines	Lesion area (%)		Lesion area (Ln)	
	WS	WW	WS	WW
6	64.80	36.20	4.19	3.62
41	0.11	0.27	0.10	0.24
8	0.39	0.08	0.33	0.08
13	0.93	0.13	0.66	0.12
43	0.11	0.01	0.10	0.01
47	0.16	0	0.15	0
Control (C2)	0	0	0	0

Table I.1 Mean lesion area developed in WS and WW trees over a 16-day period. Data are presented in both % and natural logarithm (ln) units.

Lines were ranked (Kruskal-Wallis test) according to their lesion area. There was little difference in their rankings when grown under either condition (Table I.2). The high and moderate lesion expressers (lines 6 and 41) consistently ranked highest under both moisture conditions (Table I.2).

Line rank (1=high lesions; 7=low lesions)	Line identification	
	WW	WS
1	6	6
2	41	41
3	8	43
4	13	13
5	43	8
6	47	47
7	Control	Control

Table I.2 Lines ranking based on mean leaf damage following Kruskal-Wallis test.

Statistical analysis

Effect of water deprivation on leaf damage between WS and WW trees was performed using the Student's *t*-test, SAS (1990). The output is shown below. Data analyzed are mean leaf damage (after natural logarithm transformation) for the 6 transgenic and the control lines from clone 195-529. The second *t*-test was performed without data from the high *bO*-expressing line 6.

Class TYPE: 1 = WW and 2 = WS.

TTEST Procedure

TYPE	N	Mean	SD	SE
1	7	0.58	1.34	0.51
2	7	0.79	1.51	0.57

Variances	T	DF	Prob>|T|
Unequal	-0.27	11.8	0.7888
Equal	-0.27	12.0	**0.7887**

For H_0: Variances are equal, F' = 1.27 DF = (6,6) Prob>F' = 0.78

The *t*-test under the assumptions of equal variance showed that lesion area was not significantly different between the two groups ($p < 0.7887$).

TTEST Procedure (without line 6)

TYPE	N	Mean	SD	SE
1	6	0.07	0.09	0.04
2	6	0.22	0.23	0.10

Variances	T	DF	Prob>|T|
Unequal	-1.43	6.5	0.1997
Equal	-1.43	10.0	**0.1839**

For H_0: Variances are equal, F' = 6.36 DF = (5,5) Prob>F' = 0.06

After taking out the effect of the high *bO*-expresser line 6, lesion area was still not significantly different between the two groups ($p < 0.1839$).

Conclusions

There was no difference in lesion area between the two groups, WS and WW ($p < 0.7887$). Stress due to water deprivation did not induce lesions formation in either the transgenic or control lines.

APPENDIX J: HISTOCHEMICAL AND FLUOROMETRIC GUS ASSAYS

Both assays were as described in Materials and Methods (Chapter 3). For the histochemical assays, levels of *GUS* expression were scored visually based on a scale from 0 to 5, where 0 = no blue color, 1 = low blue intensity, and 5 = high blue intensity.

CT (pMB 705)					
Line	$CuSO_4$ Concentration (μM)				
	0	5	25	50	100
1-1	5	2.5	5	5	3
3-2	5	5	5	5	5
4-3	4	3.5	5	4	3.5
5-5	5	4	5	5	5
10-7	4	5	5	5	5
20-2	5	5	5	5	5
60	5	5	5	4	5
Mean ± SE	4.7 ± 0.18	4.3 ± 0.38	5 ± 0	4.7± 0.18	4.5 ± 0.33

FT (pMB 711)					
Line	$CuSO_4$ Concentration (μM)				
	0	5	25	50	100
6	1	0	0.5	1	0.5
12-1	2	0.5	0.5	1	0.5
23	1	0.5	0.5	1	0
26	2	0	0.5	1.5	0
41	3	2	2	3	1
45	0.5	0	0	1	0
50	5	1	1.5	4	1.5
47	4	2	3	4	0.5
46	0	0	0	0	0
48	0	0	0	0	0
Mean ± SE	2.3 ± 0.57	0.8 ± 0.3	1.1 ± 0.38	2.1 ± 0.50	0.5 ± 0.20

Table J.1 Effect of copper ion concentration on histochemical *GUS* expression in CT (control) and FT (copper inducible) transgenics. Means for the FT group excluded lines 46 and 48, which had no GUS activity.

Line	$CuSO_4$ Concentration (μM)								
	0	5	25	50	60	70	80	90	100
41	3	2	2	3	2.5	2.5	2	2	1
47	4	2	3	4	3	2	2.5	2	0.5
Mean ± SE	3.5 ± 0.5	2 ± 0	2.5 ± 0.5	3.5 ± 0.5	2.8 ± 0.2	2.3 ± 0.3	2.3 ± 0.3	2 ± 0	1.3 ± 0.3

Table J.2 Effect of copper ion concentration on histochemical *GUS* expression in two FT transgenic lines.

CT Lines	with Cu (50 μM)	no added Cu
1-1	5	5
3-2	4	4
4-3	4	4
5-5	5	5
10-7	4	4
20-2	5	5
60	5	5
Mean ± SE	4.6 ± 0.20	4.6 ± 0.20

FT Lines	with Cu (50 μM)	no added Cu
6	2	3
12-1	3	3
23	3	2
26	1	2
41	4	4
45	1	1
50	5	5
47	5	5
46	0	0
48	0	0
Mean ± SE	3.0 ± 0.57	3.1 ± 0.52

Table J.3 Histochemical *GUS* expression in whole plants induced with and without copper induction (50 μM $CuSO_4$). Means for the FT group excluded lines 46 and 48, which had no GUS activity.

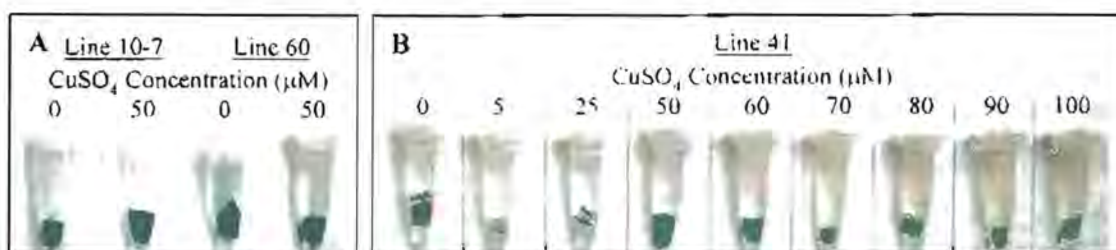

Figure J.1 Histochemical *GUS* expression in transgenic poplar with the control construct containing *GUS* but lacking the *ACE1* regulatory element (A), and the full copper induction gene-construct containing *ACE1*(B). Panel A corresponds to data presented for CT lines in Table J.3. Panel B corresponds to data for line 41 included in Table J.2.

$CuSO_4$ (μM)	Line 41		Line 47		Mean from both lines	
	Mean	SE	Mean	SE	Mean	SE
0	287	65.2	267	82.8	277	47.9
5	67	23.2	17	12.8	42	16.6
25	8	48.8	0	46.0	0	37.3
40	59	42.3	105	107.1	82	52.6
50	267	88.8	435	127.9	351	79.1
60	49	21.8	17	22.3	33	15.9
70	105	20.2	3	19.5	54	26.5
80	25	27.2	0	9.9	0	18.3
90	25	26.4	11	28.4	18	18.1
100	51	18.9	7	22.1	29	17.0

Table J.4 Mean and SE of GUS activity (pmole 4-MU/min/mg protein) in two FT lines at different $CuSO_4$ concentrations, as determined by fluorometric assay. Data are from the average of three replicates after removing background *GUS* expression, as measured in non-transgenic plants.

Statistical analysis for histochemical *GUS* expression

Analysis was performed by hand calculation using a non-parametric method (Snedecor and Cochran 1967). Data are from the histochemical *GUS* analysis on whole plants either with or without copper induction (50 μM $CuSO_4$), as shown in Table J.3, and excluding lines 46 and 48 from the FT group. Visual scores for both CT (shown in bold) and FT lines grown with or without copper induction, are arranged in increasing order by rank. Number of CT lines (n_1)=7; number of FT lines (n_2)=8.

	Visual scores and ranks for CT and FT lines when induced with 50 μM $CuSO_4$														
Score	1	1	2	3	3	**4**	**4**	**4**	4	**5**	**5**	**5**	**5**	5	5
Rank	1.5	1.5	3	4.5	4.5	**7.5**	**7.5**	**7.5**	7.5	**12.5**	**12.5**	**12.5**	**12.5**	12.5	12.5

Calculations:

T_1 = sum of ranks for the group with smaller "n" (CT)
= (7.5 x 3) + (12.5 x 4) = **72.5**

T_2 = $n_1 (n_1 + n_2 + 1) - T_1$
= 7 (7 + 8 + 1) – 72.5 = **39.5**

$T_2 < T_1$; use the smaller T = 39.5

For $n_1 = 7$ and $n_2 = 8$; 5% level of T = **38**

$T > T_{0.05}$; accept null hypothesis with $p < 0.05$

GUS activity between CT and FT groups grown under copper induction (50 μM) was not significantly different ($p < 0.05$).

	Visual scores and ranks for CT and FT lines with no added copper														
Score	1	2	2	3	3	**4**	**4**	**4**	4	**5**	**5**	**5**	**5**	5	5
Rank	1	2.5	2.5	4.5	4.5	**7.5**	**7.5**	**7.5**	7.5	**12.5**	**12.5**	**12.5**	**12.5**	12.5	12.5

Calculations:

T_1 = sum of ranks for the group with smaller "n" (CT)
= (7.5 x 3) + (12.5 x 4) = **72.5**

T_2 = $n_1 (n_1 + n_2 + 1) - T_1$
= 7 (7 + 8 + 1) – 72.5 = **39.5**

$T_2 < T_1$; use the smaller T = 39.5

For $n_1 = 7$ and $n_2 = 8$; 5% level of T = **38**

$T > T_{0.05}$; accept null hypothesis with $p < 0.05$

GUS activity between CT and FT groups grown without additional copper was not significantly different ($p < 0.05$).

Statistical analysis for fluorometric *GUS* expression

Analysis was performed using the MIXED procedure (SAS 1990). The output is shown below. *GUS* expression from two FT lines (lines 41 and 47) and a non-transgenic control were determined at different $CuSO_4$ concentrations using a fluorometric assay. Data from three replicates were transformed by taking natural logarithm. The difference between transgenic and non-transgenic expression levels (to exclude background *GUS* expression), was then analyzed.

Test of Fixed Effect

Source	NDF	DDF	Type III F	Pr > F
LINE	1	2	6.66	0.1230
COPPER	9	18	8.52	0.0001

Least-squares Means

Bold shows copper treatments that resulted in *GUS* expression significantly greater than zero.

Effect	COPPER	LSMEAN	SE	DF	t	Pr > |t|
COPPER	**0**	**1.289**	**0.202**	**18**	**6.40**	**0.0001**
COPPER	5	0.369	0.202	18	1.83	0.0836
COPPER	25	-0.190	0.202	18	-0.94	0.3575
COPPER	**40**	**0.443**	**0.202**	**18**	**2.20**	**0.0411**
COPPER	**50**	**1.460**	**0.202**	**18**	**7.25**	**0.0001**
COPPER	60	0.274	0.202	18	1.36	0.1902
COPPER	**70**	**0.460**	**0.202**	**18**	**2.28**	**0.0348**
COPPER	80	-0.075	0.202	18	-0.37	0.7129
COPPER	90	0.128	0.202	18	0.64	0.5324
COPPER	100	0.280	0.202	18	1.39	0.1819

Differences of Least-squares Means (at significance level of 0.05)

For the effect of copper concentrations, comparisons were made between the different concentrations (column 2) against no copper (column 3). *GUS* expression at 0 μM and 50 μM copper were not significantly different (shown in bold).

1	2	3	4	5	6	7	8	9
Effect	COPPER	_COPPER	Diff	SE	DF	t	Adjustment	Adj P
COPPER	5	0	-0.919	0.259	18	-3.55	Dunnett-Hsu	0.0155
COPPER	25	0	-1.479	0.259	18	-5.70	Dunnett-Hsu	0.0002
COPPER	40	0	-0.845	0.259	18	-3.26	Dunnett-Hsu	0.0281
COPPER	**50**	**0**	**0.171**	**0.259**	**18**	**0.66**	**Dunnett-Hsu**	**0.9895**
COPPER	60	0	-1.014	0.259	18	-3.91	Dunnett-Hsu	0.0071
COPPER	70	0	-0.829	0.259	18	-3.20	Dunnett-Hsu	0.0321
COPPER	80	0	-1.364	0.259	18	-5.26	Dunnett-Hsu	0.0004
COPPER	90	0	-1.160	0.259	18	-4.47	Dunnett-Hsu	0.0021
COPPER	100	0	-1.009	0.259	18	-3.89	Dunnett-Hsu	0.0075

Conclusions:

1) There was no evidence that copper induction influenced GUS activity differently between CT and FT groups ($p < 0.05$).
2) Both line 41 and 47 behaved similarly in their response toward copper induction ($p < 0.1230$).
3) Copper concentration had a strong effect on *GUS* expression in both FT lines ($p < 0.0001$). LSM analysis showed that mean *GUS* expression in transgenics grown in the presence of 0 μM and 50 μM copper were significantly different from the other concentrations. There was no difference in mean *GUS* expression between plants receiving 0 μM and 50 μM copper ($p < 0.9895$, Dunnett's test, α=0.05).

APPENDIX K: CATALOGUE OF DNA, RNA, BLOTS, PRIMERS, AND COMPUTER RESOURCES REMAINING IN THE LABORATORY

Primers for the amplification of the *bO* gene are labeled as "syntbo-L" and "syntbo-R". Primers for the amplification of the *ACE1* gene are labeled as "ACE1-F" and "ACE1-R". Primers for the amplification of the *nptII* gene are labeled as "NPTII-F" and "NPTII-R", and were provided by Dr. R. Meilan (Oregon State University). All primers are stored in a cardboard box, labeled as "Rozi - bO-copper project" and stored at –20 °C in Refrigerator #15, Richardson Hall 385. The remaining plasmid DNA of pEL301, pPAL, pWin6, pWin8, pPD5, pMB705 and pMB711 are also stored in the same box.

All plasmids are cloned into *E.coli* DH5α (Gibco BRL). Glycerol stocks are stored in Nalgene cryoboxes at –85 °C in the chest freezer (Nuaire), Richardson Hall 386. Proper documentation on their locations can be found in the laboratory "Strain Collection" inventory book in Richardson Hall 385.

Genomic DNA samples are stored in cardboard boxes. They are labeled as "Rozi-miniDNA-bO project" and "Rozi-miniDNA-copper project". Both boxes are stored at 4 °C in Refrigerator #15, Richardson Hall 385.

Total RNA samples are stored in a cardboard box, labeled as "Rozi-RNA-bO project" at –85 °C in the upright freezer (Nuaire), Richardson Hall 386.

RNA blots are kept in a metal container labeled as "Rozi-blots-bO project" at –20 °C in the lower shelf of Refrigerator # 9 ("hot fridge"), Richardson Hall 384.

A copy of this thesis is stored at: p:\tgerc\thesis\Rozi\RZthesis.doc.